A HIGH PROTEIN DIET AT THE UPPER END OF THE ACCEPTABLE MACRONUTRIENT DISTRIBUTION RANGE (AMDR) LEADS TO KIDNEY GLOMERULAR DAMAGE IN NORMAL FEMALE SPRAGUE-DAWLEY RATS

BY

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ABSTRACT

In setting the AMDR for protein at 10-35% of daily energy, the Institute of Medicine acknowledged a lack of data regarding the safety of long-term intakes. The current study assessed the impact of chronic (17 months) protein consumption at the upper end of the AMDR on renal function, histology, and inflammation.

Using plant and animal whole protein sources, female Sprague-Dawley rats (70 days old; n=8-11 at 4, 8, 12, or 17 mo.) were randomized to either a normal (NP; 15% of energy) or high protein (HP; 35% of energy) diet. Egg albumen and skim milk replaced carbohydrates in the HP diet. Diets were balanced for energy, fat, vitamins and minerals, and offered ad libitum. Renal function was analyzed by creatinine clearance and urinary protein levels. Glomerular hypertrophy, glomerulosclerosis and tubulointerstitial fibrosis were assessed on kidney sections. Kidney disease progression was determined by the measurement of transforming growth factor beta-1 (TGF-β1) and renal inflammation by the measurement of chemokines monocyte chemoattractant protein-1 (MCP-1) and regulated upon activation normal T-cell expressed and secreted (RANTES).

Rats consuming the HP compared to NP diet had ~17% higher kidney weights (P <0.0001) and ~4.8 times higher proteinuria (P <0.0001). There was a trend towards higher creatinine clearance with HP (P = 0.055). Consistent with this, HP compared to NP rats had ~22% larger glomeruli (P <0.0001) and ~33% more glomerulosclerosis (P = 0.0003). The HP diet had no significant effect on tubulointerstitial fibrosis and renal TGF-β1 levels and did not result in higher renal levels of MCP-1 and RANTES. In fact, per mg renal protein, HP rats had ~16% lower MCP-1 (P = <0.0001) and ~34% lower levels of RANTES (P = <0.0001) than NP. The absence of an increase in cytokine levels
may be a reflection of the moderate changes in renal pathology observed in rats offered HP diets.

These data in normal female rats suggest that protein intakes at the upper end of the AMDR are detrimental to kidney health in the long-term. While modest, this may have implications for individuals whose kidney function is compromised, especially given the prevalence of those unaware of their kidney disease within North America.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AMDR</td>
<td>Acceptable Macronutrient Distribution Range</td>
</tr>
<tr>
<td>BW</td>
<td>body weight</td>
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<tr>
<td>CKD</td>
<td>chronic kidney disease</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DRI</td>
<td>Dietary Reference Intakes</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>ESRD</td>
<td>end-stage renal disease</td>
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<td>GFR</td>
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<td>inhibitory subunit-κB</td>
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<td>IOM</td>
<td>Institute of Medicine</td>
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<td>K/DOQI</td>
<td>Kidney Disease Outcome Quality Initiative</td>
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1. INTRODUCTION AND RATIONALE FOR THESIS RESEARCH

The benefits or adverse health consequences of a long-term or chronic high-protein (HP) diet are unclear and misunderstood, despite on-going media exposure. HP diets continue to be used as a means of weight loss and weight control in overweight and obese individuals. Unfortunately, there is little published research supporting the safety of these diets. The Institute of Medicine’s (IOM) report on Dietary reference Intakes (DRI) for Macronutrients (IOM, 2002) states:

“There was insufficient evidence to suggest an Upper Level for protein and insufficient data to suggest an upper limit for an AMDR (Acceptable Macronutrient Distribution Range) for protein. To complement the AMDRs for fat and carbohydrate for adults, protein intakes may range from 10 to 35 percent of energy intake to ensure a nutritionally adequate diet” (p 661), and

“Research is needed on high protein intakes (>145 mg N/kg/day) in relationship to: positive nitrogen balance and requirement estimates; metabolic and possible toxic effects…and pathways impacted by these high intakes” (p 573).

This substantial change in the dietary range was made despite the acknowledged lack of data on the long-term effects of a protein intake at the high end of this range. The inclusion of the AMDR suggests that protein intake within this range poses no danger to health. However, careful reading of this report reveals that the data supporting this is sparse and the perception is one of approval for HP diets. The previous Recommended Nutrient Intake for adult Canadians suggested that 13 to 15% of daily energy should come from protein (Health Canada, 1990). The AMDR recommendation is also at odds with the World Health Organization (WHO) that recommends that protein intake should be between 10 to 15% of energy (WHO, 2003). However, dietary intake data suggests that few individuals consume protein levels at the high end of the AMDR. Approximately 1% of individuals consume more than 23% of their energy as protein or ~300,000
Canadians (IOM, 2002). These numbers are likely higher since most individuals underreport their protein intakes (Subar et al, 2003; Rosell et al, 2003). Consequently, new recommendations that support higher levels of protein intake than the past could change the dietary patterns of adults. This question is particularly relevant due to the current popularity and publicity supporting the safety of HP diets as an accepted means of weight management (Bravata et al, 2003; Foster et al 2003; Samaha et al, 2003).

The life expectancy for Canadians has increased due to advancements in health care and technology with males living to ~77 and females living to ~82 years of age (Statistics Canada, 2004). According to the Kidney Foundation of Canada (KFC), the number of Canadians with end-stage renal disease (ESRD) requiring dialysis increases by 8 to 10% per year (KFC, 2003). In 2004, ~19,000 individuals were on dialysis and this number is expected to double by 2014 (KFC, 2007). Approximately 80% of these individuals are on hemodialysis which costs ~$70,000/year/person (McFarlane et al, 2002; Lee et al, 2002) with an estimated annual health care cost of $1.3 billion. This increased longevity combined with an escalating incidence of renal disease, presents Canada with an ever-expanding demand for dialysis and kidney transplantation.

Yet, less than one-quarter of people with test results indicating chronic kidney disease (CKD) are aware they have kidney problems (The American Society of Nephrology, 2004). Further to this, 90% of individuals with moderately decreased kidney function have reported never being told of the compromise that was occurring to their kidneys and that they were beginning to fail (Coresh et al, 2005). These statistics demonstrate that CKD is often under diagnosed and under treated in the clinical population. As kidney function declines, complications develop in relation to fluid
overload, electrolyte and acid-base imbalances, and the accumulation of nitrogenous wastes (Snively and Gutierrez, 2004). Mild renal insufficiency is also associated with an increased risk for cardiovascular disease (CVD), despite controversy (Culleton and Hemmelgarn, 2003; Garg et al, 2002; Henry et al, 2002). Therefore, it is important to not only gain information on the safety of a long-term HP diet for healthy individuals, but also for those with mild renal insufficiency. The unmeasured economic and humanitarian burden and the public health implications caused by unsafe protein recommendations would far exceed these alarming statistics.

1.1 Literature Review

1.1.1 Renal Disease Definition and Classification

The National Kidney Foundation (NKF) defines CKD as kidney damage or a glomerular filtration rate (GFR) of less than 60 ml per minute per 1.73 m$^2$ for three or more months (Kidney Disease Outcome Quality Initiative [K/DOQI], 2002). Below this level, the possibility of future complications of CKD increases. In persons 30 years or younger, the normal GFR is 125 ml per minute per 1.73 m$^2$ however, after the age of 30, GFR declines by 1 ml per minute per 1.73 m$^2$ a year (Snively and Gutierrez, 2004).

In February 2002, the NKF K/DOQI published 15 clinical guidelines on CKD (K/DOQI, 2002). The goals of these guidelines were to 1) define and classify the stages of CKD, despite the basis for disease; 2) consider which laboratory measurements best suit the clinical assessment; 3) link together the level of renal function with CKD complications; and 4) classify the risk for loss of kidney function and development of CVD (K/DOQI, 2002).
The K/DOQI separated CKD into five stages based on GFR and metabolic consequences: stage 1 involves kidney damage with normal or increased GFR (≥90), stage 2 is kidney damage with mildly decreased GFR (60-89), stage 3 is moderately decreased GFR (30-59), stage 4 is severely decreased GFR (15-29) and stage 5 equals kidney failure (<15) or dialysis (K/DOQI, 2002).

1.1.2 Prevalence and Economic Cost

CKD is a public health problem in North America that can develop into further medical complications. Data derived from the third National Health and Nutrition Examination Survey (NHANES III) estimates that 11% of the general American adult population (19 million) has some degree of CKD (Coresh et al, 2003). Assuming a similar prevalence in Canada, an estimated 2 million Canadians could have some degree of CKD. The estimated medical and economic costs of CKD are expected to reach $28 billion (US) annually by 2010, with an additional $90 billion (US) in annual costs related to associated increased CVD, infections, and hospitalizations (Excerpts from the United States Renal Data System, 2000). Without interventions, many of these patients may develop end stage renal disease (ESRD) which requires long-term dialysis or kidney transplantation.

Globally ESRD affects approximately 750,000 patients (Klahr, 2001). The United States (US) has the highest incidence of ESRD with 320 people per million, followed by Japan with 250 million people per million and Canada somewhere in the middle of the world at 140 per million (KFC, 2005). According to the Canadian Institute for Health Information (CIHI), the number of ESRD patients has risen from 13 per 100,000 in 1997 to 16 per 100,000 or a ~20% increase over 5 years (CIHI, 2004). ESRD is used in the US
for administrative purposes and indicates that a patient is being treated with dialysis or kidney transplantation, but does not define a specific level of kidney function or even failure (Levey et al, 2003).

1.1.3 Prevalence Based on Stage

Based on CKD stage, 5.9 million adult Americans have stage 1, 5.3 million have stage 2, 7.6 million are at stage 3, and 400,000 have severely decreased GFR and are at stage 4 (Coresh et al, 2003). The prevalence of dialysis or stage 5 is estimated to be 300,000 and is derived from the US Renal Data Survey (United States Renal Data System 1998 Annual Report). See Table 1 for a summary of these five stages and their prevalence within the US.
<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
<th>GFR$^a$</th>
<th>Prevalence$^b$ (%)$^c$</th>
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<td>-</td>
<td>Increased risk for CKD</td>
<td>&gt;60</td>
<td>d</td>
</tr>
<tr>
<td>1</td>
<td>Kidney damage with normal or elevated GFR</td>
<td>≥ 90</td>
<td>5.9 (3.3)</td>
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<tr>
<td>2</td>
<td>Kidney damage with mildly decreased GFR</td>
<td>60 - 89</td>
<td>5.3 (3.0)</td>
</tr>
<tr>
<td>3</td>
<td>Moderately decreased GFR</td>
<td>30 – 59</td>
<td>7.6 (4.3)</td>
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<td>4</td>
<td>Severely decreased GFR</td>
<td>15 – 29</td>
<td>0.4 (0.2)</td>
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<tr>
<td>5</td>
<td>Kidney failure (dialysis)</td>
<td>&lt; 15</td>
<td>0.3 (0.1)</td>
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NKF = National Kidney Foundation; GFR = glomerular filtration rate; CKD = chronic kidney disease.

$^a$GFR expressed in ml per minute per 1.73 m$^2$

$^b$Estimated United States adult prevalence expressed in millions per CKD stage

$^c$Estimated percentage of United States adult population per CKD stage

$^d$Prevalence of persons at increased risk for CKD has not been estimated accurately
1.1.4 Treatment Based on Stage

Measurement of renal function is crucial in the early diagnosis and management of renal disease. During stage 1 and 2 when GFR is either increased or mildly decreased, the treatment of comorbid conditions, strategies to slow CKD progression and ways to reduce CVD risk begins (Levey et al, 2003). When GFR becomes significantly decreased (stage 4) the patient is prepared for kidney replacement therapy to decrease the likelihood of organ failure (Levey et al, 2003). By the time GFR reaches < 15 ml/min (stage 5) with uremic symptoms the subject is prepared for dialysis and eventual transplantation (Levey et al, 2003).

1.1.5 Disease Assessment

GFR assessment continues to be the single most useful and widespread method to determine renal function, health, and disease. Estimates of GFR stem from serum creatinine and prediction equations that offset age, sex, ethnicity, and body size. Serum creatinine should not be used on its own due to short-term variations in tubular secretion, extra-renal excretion, and generation (Shemesh et al, 1985; Perrone et al, 1992). A useful estimate of GFR is provided by The Modification of Diet in Renal Disease (MDRD) study equation (K/DOQI, 2002):

\[
GFR = 1.86 \times \text{serum creatinine in mg/dl}^{-1.154} \times \text{age}^{-0.203} \times (0.742 \text{ if female}) \times (1.21 \text{ if African American})
\]

Renal function can also be estimated through serum creatinine using the Cockcroft-Gault formula for creatine clearance (CCr)(Cockcroft and Gault, 1976):

\[
\text{Cockcroft-Gault CCr} = \frac{(140 - \text{age})}{\text{serum creatinine}} \times (\text{weight} \div 72) \times (0.85 \text{ for women}) \times \text{BSA} \div 1.73 \text{ m}^2
\]
where BSA is body surface area and is estimated using the following formula (Du Bois and Du Bois, 1916):

\[
BSA (m^2) = 0.20247 \times \text{height (m)}^{0.725} \times \text{weight (kg)}^{0.425}
\]

The use of creatinine to calculate GFR is an estimate at best. In an ideal clinical setting, markers that are freely filtered at the glomerulus, cannot be metabolized, secreted or absorbed at the tubules, and enters urine quickly would be the most accurate (Frennby and Sterner, 2002). Inulin remains the classic example of this (Gaspari et al, 1998), but is clinically impractical due to expense, inconvenience, catheterization, and precise timing during urine collection (Frennby and Sterner, 2002).

More essential and important than an absolute perfect GFR filtration marker is early detection to initiate treatment, prevent adverse outcomes, and delay further progression of CKD. Otherwise the likelihood of premature mortality is increased two- to threefold, dialysis risk becomes elevated, and the development of congestive heart failure or other cardiovascular events rises (Collins et al, 2003).

A persistent increase in urinary protein excretion or proteinuria is a reliable marker for CKD progression (Iseki et al, 2003; Remuzzi and Bertani, 1998). Healthy individuals usually excrete less than 150 to 200 mg/d of protein in the urine, 10-20 mg/d of which is albumin (Schrier, 2001). Some controversy exists regarding proteinuria as a risk factor for clinical nephropathies, yet when urinary protein excretion is >3-5 g/24 h, these individuals have a greater renal disease progression rate than those who excrete <1-3 g urinary protein/24 h (Remuzzi and Bertani, 1998).

In addition, increased excretion of the protein albumin is a sensitive marker for CKD and a strong predictor of ESRD (Levey et al, 2003; Guidelines Subcommittee,
Albuminuria is an increased urinary excretion of albumin, and microalbuminuria (MA) is albumin excretion above its normal range of 10-20 mg/d, but below the minimum detectable level for total urinary proteins (Levey et al, 2003). MA is defined as 30-300 mg/d and is a principal risk factor for progressive renal failure, especially in those with diabetic nephropathy and hypertension (de Jong et al, 2002). In those without renal disease, MA indicates general endothelial dysfunction (Wrone et al, 2003) and is associated with an increased risk of CVD. In the US, approximately 6% of men and 10% of women have microalbuminuria (Jones et al, 2002). Proteinuria and albuminuria are used interchangeably in medicine, and the protein albumin is highest in the urine of those with renal disease (Venkat, 2004).

1.2 Pathology and Pathophysiology of Renal Disease

1.2.1 The Glomerulus

The glomerulus is the initial site of plasma filtration at the nephron. The basement membrane of the glomerulus is porous as to allow passage of low-molecular-weight solutes and water (Klahr et al, 1988). Under normal circumstances the daily passage of proteins does not include albumin, but sometimes it can be found in urine.

The foot processes of podocytes cover the external surface of the glomerular basement membrane that act as a filtration barrier and control membrane turnover under normal conditions (Kerjaschki, 2001; Kriz et al, 1998α; Pavenstädt, 2003). In renal diseases characterized by proteinuria these foot processes can fuse, and compromise filtration function (Klahr et al, 1988).

Mesangial cells of the glomerulus can generate prostaglandins, produce and break down the basement membrane, synthesize cytokines, produce platelet-derived growth
factor, epidermal growth factor, and interleukin-1 (Schlondorff, 1987). These cells can contribute to histological changes of the glomerular basement membrane via proliferation, remodeling, and extracellular matrix (ECM) synthesis (Klahr et al, 1988). Ultimately, this could lead to sclerosis and loss of numerous filtering glomeruli.

1.2.2 Renal Disease Progression

The progression of renal disease can be affected by several factors such as hyperfiltration, glomerular hypertrophy, glomerulosclerosis, and proteinuria. The initial injury and mechanisms may vary, but the end result of renal fibrosis is a common histological feature. There are several bioactive factors implicated in this initiation process which include cytokines, growth factors, angiotensin II, endothelin, T cells, monocyte/macrophages, and platelets (Remuzzi et al, 1997; Klahr et al, 1988).

Progressive glomerulosclerosis is associated with aging and by the age of 80, 40% of glomeruli exhibit a certain degree of sclerosis (Kaplan et al, 1975). In humans and animals with CKD, glomerulosclerosis is characterized by the progressive involvement of segments within glomeruli, a decrease in number, and tubular destruction (Klahr, 2001). These histological changes are correlated with an impairment in glomerular and tubular function. On electron micrographs, the tubular and glomerular basement membranes are thickened early in disease and become condensed and deformed masses with further progression (Klahr et al, 1988).

Histologically, progressive CKD is characterized by the accumulation of ECM within the renal interstitium and progressive tubular atrophy (Klahr et al, 1988). Normal renal structures become scarred and replaced with fibrotic tissues. This impairs renal function and leads to eventual kidney failure.
1.3 **Protein Restriction and Renal Disease**

Dietary protein restriction slows the progression of renal disease by normalizing glomerular hemodynamics (Brenner et al 1982; Hostetter et al, 1986). In rats with renal ablation, hyperfiltration can be attenuated with a low-protein diet, and renal disease progression halted or delayed (Brenner et al 1982; Hostetter et al, 1981; Zatz, 1996). Since then protein restriction has been considered a strategy for slowing renal disease progression. Aukema and colleagues (Aukema et al, 1992; Aukema et al, 1999; Ogborn and Sareen, 1995; Tomobe et al, 1994) have demonstrated that in animal models of renal disease characterized by renal inflammation and fibrosis, diets with 5-8% protein as energy significantly slowed renal disease.

However, it has been difficult to document this renoprotective effect in humans and this topic remains under some controversy. The MDRD study was designed to clarify the role of protein restriction in CKD. Originally there was a lack of conclusive evidence, but following secondary analyses and several meta-analyses the supervised low-protein diet of 0.6–0.75 g/kg/day benefited those whose GFR <25 ml/min (Klahr et al, 1994; Levey et al, 1996; Pedrini et al, 1996). The NFK recommends a planned low-protein diet in CKD patients not on dialysis to assist with the improvement of metabolic complications, preserve nutritional status, and slow kidney disease progression (K/DOQI, 2002). With a low-protein diet a major concern is nutritional adequacy. Yet, in the MDRD study both the low-protein and very-low protein diets were appropriate under supervision of a qualified dietician (Klahr et al, 1994).
1.4 Prevalence of Obesity

The incidence of obesity has rapidly increased over the last two decades resulting in a national epidemic. Approximately 33% of adult Canadians are overweight while 15% are obese (Statistics Canada, 2002). In 1997, the total Canadian health care cost attributable to obesity was estimated to be greater than $1.8 billion or 2.4% of the total expenditure on diseases (Birmingham et al, 1999). National studies in the US indicate that 56% of the adult population is overweight, and 20% of all Americans are obese (Rippe et al, 1998). The vast majority of overweight and obese individuals are at a greater than average risk of developing comorbid conditions such as type 2 diabetes, CVD, hypertension, stroke, gallbladder disease, sleep apnea, and respiratory problems (Pi-Sunyer, 1993). For those that are obese, >70% have at least 1 established comorbid condition (US Department of Health and Human Services, 1990). The impact of at least one condition with respect to the Health Care System is substantial. This further demonstrates the significance for the deployment of a safe and successful strategy against obesity.

1.5 Link between Obesity and Renal Disease: The Metabolic Syndrome

Obesity plays an important part in the progression of kidney disease. It is also the phenotypic hallmark of the metabolic syndrome (insulin resistance syndrome) that is characterized by hyperglycemia, hypertriglyceridemia, dyslipidemia, hypertension, and hyperinsulinemia (Qiao et al, 2007; Reaven, 1988). The prevalence of this syndrome continues to rise within the US with approximately 47 million individuals afflicted (Coresh et al, 2003). The metabolic syndrome contributes to the development of type 2 diabetes, hypertension, and CVD which are its ties to renal disease. (DeFronzo and
The question remains whether or not these links are to renal disease are caused by obesity or are simply associations.

Obesity has also been connected with the initiation and progression of glomerulonephritides (de Jong et al, 2002; Kambham et al, 2001) and higher incidences of focal and segmental glomerulosclerosis (Verani, 1992). Focal refers to the scarring of some of the glomeruli, while others remain intact. Segmental is damage to a portion of an individual glomerulus. However, this may be difficult to determine since obesity is also a risk factor for hypertension and diabetes (Rosenbaum et al, 1997). Recently, the incidence of kidney disease has increased alongside obesity (Praga, 2002).

Hoehner et al (2002) correlated the metabolic syndrome profile with MA in a cross sectional study of Native Americans from Minnesota and Wisconsin. After being stratified, those with ≥3 syndrome traits had a 2.3 fold increased odds of having MA when compared to the control group without the syndrome. Both Palaniappan et al (2003) and Chen et al (2004) have extracted data from NHANES III database and discovered this association. Chen et al (2004) also discovered a significant correlation between metabolic syndrome risk factors and a GFR <60 ml/min. The two traits which posed the greatest risk were hypertension and hyperglycemia (Chen et al, 2004). More research is required to investigate the potential role of obesity and the progression of CKD since the prevalence of both of these conditions continues to rise.

1.6 Weight Loss Strategies

Billions of dollars are spent each year on weight loss diets and products with new diets constantly appearing on the market. At any given time, approximately 45% of women and 30% of men in the US are trying to lose weight (Serdula et al, 1999). Within
North America, women diet to hopefully achieve a societal sanctioned slim body size (Trottier et al, 2005). As a result, women are the primary target of popular diet strategies. Unfortunately, few individuals maintain their weight loss after trying diet programs. Researchers estimate that 80 to 85% of dieters who lose weight will gain it back within 1 to 5 years (Pi-Sunyer, 1999). These diets may fail due to food restriction and quantity, or the purchase of special foods which becomes costly and repetitive. These low achievement rates could also be due to a personal inability to adhere to a long-term weight loss strategy.

1.6.1 Low-carbohydrate, HP Diet

Low-carbohydrate diets continue to exist as a means of quick weight loss despite their unknown long-term efficacy and safety. In the 1860’s, William Banting popularized the first low-carbohydrate diet and claimed he was never hungry and lost 46 pounds in one year (Banting, 1869). The late cardiologist Robert C. Atkins re-popularized the diet in the 1970’s, and in the beginning of the 21st Century re-promoted the diet in North America. Other low-carbohydrate HP diet books have spawned including South Beach, Protein Power, and Sugar Busters. Each of these diets differ in composition, with emphasis on a higher than recommended protein intake and the restriction of food choices, mainly carbohydrates.

1.6.2 Theory behind the Diet

Advocates of these diets claim that higher protein and lower carbohydrate intakes promote the metabolism of adipose tissue in the absence of dietary carbohydrate resulting in rapid weight loss without significant long-term health effects (Atkins, 2002). Protein intakes as percent of daily calories with the Zone, Atkins, and South Beach diets can be
34, 27, and 23%, respectively. On a 2000 kcal/d diet this is 170, 135, and 115 g/d of protein, respectively. Typical low-carbohydrate recommendations range from 20 to 90 g of carbohydrate a day which based on a 2000 kcal/d diet is ~4 to 18% of daily calories (Atkins, 2002). Intakes this low mimic the body’s reaction to prolonged fasting, in which glucose concentrations become low and there is a switch to ketones derived from fatty acids as the preferred energy source (Pi-Sunyer, 1999). Heart and muscle tissue use ketones to spare the limited glucose supply required for brain function. Caution exists regarding low-carbohydrate diets because an accumulation of ketones may result in abnormal metabolism of insulin and impaired liver and kidney function (Bravata et al, 2003). Therefore, in those with CVD, type 2 diabetes, dyslipidemia, impaired renal function, or hypertension there is increased concern.

The primary reason for the initial rapid weight loss is diuresis, caused by low-carbohydrate intake and its effects on sodium loss, water loss, and glycogen depletion (Tapper-Gardzina et al, 2002). Also, by restricting carbohydrate and replacing it with protein, satiety occurs.

There is a scarcity of data addressing popular diets and research has yet to publish data concerning renal function and the long-term effects of low-carbohydrate HP diet. The public remains largely uneducated on the safety of these diet programs and the absence of sufficient long-term research makes it difficult to ascertain the potential impacts on health.

1.7 Effect of High Dietary Protein on the Normal Kidney

It has been known for some time that a HP diet induces renal hypertrophy (Osborne et al, 1926; Wilson 1933; MacKay et al, 1928; Kenner et al, 1985; Kaysen et al,
1989) and increases GFR in both acute and chronic HP feeding (Brenner et al, 1982; Pullman et al, 1954; Kaysen et al, 1989; Pitts, 1944). Consequently, an increase in GFR does not represent an improvement in renal function, but is actually a physiologic recruitment of renal functional reserve to offset the increased burden of a HP diet (Brenner et al, 1982). Animal organ systems possess a functional capacity that can handle 1-10 times the average load placed upon them by normal physiological demands (Alexander, 1981; Weibel et al, 1991).This safety factor can be used in periods of increased demand to the system, like HP feeding and renal filtration.

Magnusson et al (1990) illustrated this alteration in the permselectivity of the glomerulus with different sized polyethylene glycols administered intravenously to rats after given 8 and 22% dietary protein. In the normal rats on the higher protein diet there was an increase in the amount of polyethylene glycols recovered in the urine. Bouby et al (1988) measured renal function in rats fed a low protein diet that contained 10% casein or a HP diet that was 32% casein for 5 to 6 weeks. Renal hypertrophy occurred in the HP animals and GFR as measured by creatinine clearance in the HP rats was almost double that of the LP rats.

At a cellular level, renal hypertrophy is cell enlargement and not cell multiplication. There is an increase in protein per cell, protein per DNA, and cell size (Fine and Norman, 1989). The link between renal hypertrophy and HP diets is not fully understood. It has been suggested that an increase in urinary concentration activity may be the cause of nephron hypertrophy (Bouby et al, 1988). In the Western diet ~40-50% of urinary solutes are urea and in laboratory rats and mice excretion can be at this level or substantially higher based on diet composition (Yang and Bankir, 2005). Most rodents
especially mice concentrate their urine more so than humans which could expedite the effect.

1.7.1 Human studies on GFR

In humans, Pullman et al (1954) measured GFR by inulin clearance and renal plasma flow via Diodrast. One of three diets was ingested for 2 weeks consisting of 2.3 to 3.0, 1.0 to 1.4 or 0.1 to 0.4 g protein/kg body weight. The HP diet group had a GFR increase of 13.1 ml/min per 1.73 m$^2$ when compared to the medium protein group. The mean increase in GFR for the medium compared to the LP diet group was 9.0 ml/min per 1.73 m$^2$. As for renal plasma flow, a progressive increase was observed as protein intake increased. Rodríguez-Iturbe et al (1988) also measured GFR via inulin clearance and renal plasma flow by PAH clearance in humans using a meat meal that contained either 1.35 g of protein, 1.08 g of protein or 0.55 g of protein per kg/body weight. The control was a carbohydrate meal providing <15 g of total vegetable protein. After 30 min GFR began to rise after the meat meal and peaked at 120 min with no effect seen with the control diet. The peak increase in GFR above baseline was dose dependent being 85%, 53% and 7.5% with the large, moderate and mild protein level, respectively.

1.7.2 Long-term HP Diets and Rats

When normal rats are given long-term HP diets they exhibit increased proteinuria and worsened renal pathology. Bertani et al (1989) examined the effects of 7, 23 and 42% protein as energy in normal Sprague-Dawley rats over 20 months starting at 66-70 days. A strong correlation between proteinuria and renal lesions was found. Rats on the highest protein diet developed proteinuria and the average proteinuria was higher than rats given 23% protein as energy. They also developed higher serum creatinine by the end of the
study compared to the other two diets that remained unchanged over time. Of the 10 rats on the highest protein diet, all had tubulo-interstitial damage and focal segmental glomerular sclerosis. Whereas, 6 out of the 10 rats on the 23% diet and 3 out of 10 on the 7% diet had any histological damage.

1.7.3 Rat Studies and Moderate Protein Levels

In moderately elevated levels of dietary protein, adverse effects have been observed in long-term studies. Rao and colleagues (Rao et al, 1993; Rao et al, 2001; Rao 2002) fed Fischer rats either 14-15% or 23% protein by weight for 2 years. Diets differed in protein ingredient sources to achieve the acquired levels, but were formulated to have similar nutrient content. In the rats fed 23% protein, renal pathology scores were 40% higher compared to the 14-15% diet group. Prior to these studies, Masoro and colleagues (Yu et al, 1985; Maeda et al, 1985; Masoro et al, 1989) reported similar results in several studies that examined the effect of protein restriction on nephropathy and lifespan in Fisher rats.

When 52% protein as energy was fed to healthy Wistar male rats for 6 months, Lacroix et al (2004) found no significant adverse effects on renal function and pathology. Although renal toxicity was absent, GFR was not examined or other early markers of progressive kidney damage. In contrast, Hostetter et al (1986) did find evidence of glomerulosclerosis and proteinuria in normal rats fed a HP diet containing 40% protein by weight as casein for 4 and 8 months. These control rats were compared with unilaterally nephrectomized rats, and with one and one-third nephrectomized rats. At both time intervals it was found that with increasing degrees of renal ablation, HP diets
resulted in several fold increases in proteinuria and glomerulosclerosis (Hostetter et al, 1986).

1.7.4 Long-term Human Studies and HP Diets

The research regarding the long-term effects of a HP diet in human kidneys at the upper end of the AMDR is lacking. The few reports on moderately HP diets in humans with normal kidneys are conflicting. Hoogeveen et al (1998) found that a 0.1 g/kg increase in dietary protein was associated with an increase in MA. They found this association even though <5% of the population studied (680, 50 to 75 year old Caucasians) had protein intakes >1.5 g/kg body weight which is considered moderately high (~20% of daily energy). In contrast to these findings, Skov et al (1999) reported that protein level did not alter albumin excretion in overweight subjects. This study lasted 6 months, but the protein levels achieved were adequate at 14% and moderately high with dietary protein at 22% of daily energy.

More current to this, analyses of the Nurses’ Health Study demonstrated no effect of protein level on renal function in women with normal renal function at baseline (GFR>80 ml/min per 1.73 m²)(Knight et al, 2003). This 11 year study consisted of 1624 women ages 42 to 68 at baseline. The upper quintile of protein intake ranged from 87-164 g/kg/day, meaning very few were consuming protein in the upper range of the AMDR. The 99th percentile of women in this age range consume 105 g per day (IOM, 2002), with a similar distribution approximately 1% of the women in this study would be consuming greater than 20% energy as protein. Unfortunately, this study does not include a sufficient number of subjects at the upper end of the AMDR for protein to adequately determine the affect on normal kidneys.
1.8 Effect of High Dietary Protein in Mild Renal Insufficiency

Even if protein intake at the upper end of the AMDR were to have no adverse effects in healthy individuals with normal renal function, there is still a significant portion of the population with mildly compromised kidneys for which HP diets may have adverse effects. Based on data from NHANES III, 23-32% of adults >20 years of age have mild renal insufficiency and 41-47% of people >40 years of age have a GFR between 60 and 89 (Coresh et al, 2003). These individuals are considered healthy and are often unaware of their condition. Estimates of CKD prevalence from health maintenance organizations, reported that 1/3 to 1/2 of those with CKD are unaware of their condition (Obrador et al, 2002). A study by Jurkovitz et al (2002) on a population of individuals at an increased risk for CKD based on family history of ESRD, found that only 13% of those screened for CKD were aware of their disease. Therefore, these recommendations remain relevant since a significant portion of the population has mild renal insufficiency.

The data in humans regarding HP intakes and mild renal insufficiency are inconclusive. However, Knight et al (2003) found that in women with mild renal insufficiency whose GFR was between 55 and 80 ml/min, each 10 g increase in protein was associated with a decrease in GFR by 7.72 ml/min per 1.73 m$^2$. Women in the highest quintile had a 3.51 times higher risk of at least a 15% decrease in GFR when compared to the lowest quintile. This is significant since 30% of the participants in this study were classified as having mild renal insufficiency.

Unfortunately, the kidney undergoes extensive histologic damage well before there is any significant change to renal filtration capacity. Mild renal insufficiency does not have any apparent symptoms, yet puts one at an increased risk for the CKD similar to
the association between atherosclerosis and heart disease. Therefore, since a significant portion of the North American population with mild renal insufficiency are considered healthy in the context of the DRI recommendations, they may be adversely affected by a protein intake at the upper end of the AMDR.

### 1.9 Effect of High Dietary Protein in Renal Disease

The majority of the information regarding HP diets and the effects on the kidney is based on studies in established renal diseases. This information may be relevant to the effects protein may have on normal kidneys. Human studies have demonstrated that LP diets can help slow the progression of established renal disease. Within these studies, reducing the protein intake to an adequate, but low level slows progression and is associated with reduced mortality. LP diets and renal disease progression remains a controversial topic due to the initial report from the MDRD study. It was concluded that dietary protein did not affect renal disease progression (Klahr et al, 1995). Reanalysis of the data using actual intakes rather than those prescribed revealed a beneficial effect (Levey et al, 1999). Further meta-analyses support the benefit of reduced protein intakes in chronic renal diseases (Kasiske et al, 1998; Pedrini et al, 1996; Fouque et al, 2000).

Since renal disease can progress slowly, the main barrier to examining HP intakes in established renal disease is the length of time required to follow patients. An animal model such as a rodent affords the opportunity to address the long-term safety of a HP diet. In rodent studies characterized by renal inflammation and fibrosis, Aukema and colleagues have demonstrated that diets with 5-8% of energy as protein compared to 20-21% significantly slowed disease progression (Aukema et al, 1992; Aukema, et al 1999; Tomobe et al, 1994). The benefit of protein restriction in animal models of renal disease
has been demonstrated by other studies (Klahr et al, 1988; Maroni and Mitch, 1997; Zeller, 1991). Although the effect of HP on renal disease has not been thoroughly studied in humans, rodent data exists. The severity of disease progression was significantly increased when rats were provided 36-37% compared to 12-14% of the diet as protein (Yanagisawa et al, 1998; Kenner et al, 1985; De Keijzer et al, 1990). In fact, these dietary protein levels are very close to the upper end of the AMDR recommendations.

1.10 Mechanisms of High Dietary Protein Intake and Progressive Renal Disease

In normal and compromised kidneys, high dietary protein in the short term increases GFR, hyperfiltration, and proteinuria (Kim and Linkswiler, 1979; Hegsted et al, 1981; Allen et al, 1979; Hegsted and Linkswiler, 1981; Brenner et al, 1982; Brenner et al, 1996; Schuette et al, 1980; Hostetter et al, 1986). Consequently, these alterations in renal hemodynamics alter the permselective properties of the glomerulus and proteins like albumin can join ultrafiltrate and be excreted in urine. Therefore, it is also possible for growth factors from the blood to reach the tubules where they can either activate fibroblast proliferation to myofibroblasts and ECM deposition directly (Hirschberg and Wang, 2005). Filtered growth factors can also trigger a basolateral chemokine response at the tubules which encourages macrophages to initiate an indirect fibrogenic TGF-β signal (Hirschberg and Wang, 2005). In addition, proinflammatory proteins may also reach proximal tubules due to production at the injured glomerulus (Hirschberg and Wang, 2005; Eddy, 2000).

In chronic nephropathies glomerular hypertension leads to increased glomerular permeability, protein filtration, and urinary proteins (Campbell et al, 2002). This glomerular damage produces a protein rich ultrafiltrate that when reabsorbed at the
proximal tubules via endocytosis initiates damage, interstitial inflammation, and scarring (Remuzzi and Bertani, 1998; Remuzzi et al, 1997). This can result in progression towards tubulointerstitial fibrosis, renal insufficiency, and loss of function. This sequence of protein toxicity is common to rats with toxic or immune proteinuric nephropathy (Bertani et al, 1986). When rats are given intravenous albumin injections, excess glomerular filtration results in renal scarring in a similar pathway (Eddy, 1989).

In experimental models of renal disease, the degree of proteinuria is correlated to the magnitude of renal damage (Remuzzi and Bertani, 1998). This overexposure to filtered proteins up-regulates genes for vasoactive and inflammatory mediators such as endothelin-1 (ET-1) and monocyte chemoattractant protein-1 (MCP-1)(Zoja et al, 1995; Wang et al, 1997). In renal diseases, the infiltration of monocytes, macrophages, and T cells is thought to play a central role in progressive interstitial fibrosis and renal failure (Bohle et al, 1992). Monocytes can stimulate the proliferation of fibroblasts, synthesis of collagen, and ECM deposition through the release of transforming growth factor beta-1 (TGF-β1)(Border and Noble, 1994; Roberts et al, 1992; Eddy, 1996). The transformation of a fibroblast to a myofibroblast is controlled by cytokines, mechanical factors, and growth factors like TGF-β1 resulting in collagen production (Swaney et al, 2005). Ultimately, fibrosis of the tubules and interstitium leads to loss of renal function. Please see Figure 1 for a schematic representation as demonstrated by tubulointerstitial fibrosis progression.
Figure 1. Schematic representation of the stages of tubulointerstitial fibrosis. TBM = tubular basement membrane; ECM = extracellular matrix. (Adapted from The Lancet, 365, El Nahas and Bello, Chronic kidney disease: The global challenge, 1553-63, 2005, with permission from Elsevier 2007).
1.11 Early Inflammatory Markers in Renal Disease

As previously described, protein overload can result in the excessive tubular reabsorption and secretion of vasoactive mediators, growth factors, and chemokines. Examples of the above include ET-1, transforming TGF-β1, MCP-1, and regulated upon activation normal T-cell expressed and secreted (RANTES). These mediators activate tubular epithelial cells that in turn induce cellular infiltration and inflammation of the interstitium, ultimately causing fibrosis, tubular cell hypertrophy, and renal scarring (Matsuo et al, 2003; Remuzzi and Bertani, 1998; Inan et al, 2003; Tamaki et al, 2003; Zeisberg et al, 2002; Zoja et al, 2003; Yokoyama et al, 2003). Inhibition of these chemokines and cytokines in animal models of renal disease demonstrate their importance in the early stages of renal fibrosis, inflammation and hyperproliferation (Yokoyama et al, 2003; Wang and Hirschberg, 2000; Shimizu et al, 2003, Goumenos et al, 2002; Benigni et al, 2003). The importance of these factors was demonstrated by the appearance of these chemokines and cytokines in urine and their subsequent disappearance during the prevention of proteinuria and renal disease (Yokoyama et al, 2003; Gilbert et al, 2001; Morii et al, 2003).

The above are important markers for renal disease especially during its early progression and are the primary focus of interest in the current study. This does not mean that other agents of inflammation are not critical; instead they are possible directions for future research.

1.11.1 Nuclear Factor-κB

Nuclear factor-κB (NF-κB) is a ubiquitous transcription factor involved in the immune and inflammatory responses of mammals. NF-κB exists as either a homodimer
or heterodimer in a wide variety of subunits, but the most abundant and studied form of NF-κB is the p50/p65 heterodimer (Inan et al, 2003).

NF-κB is usually found inactive in the cytoplasm of unstimulated cells bound to inhibitory subunit (IκB), an inhibitory protein (Baldwin, 1996; Barnes and Karin, 1997; Baeuerle, 1998). Upon stimulation, NF-κB is released from IκB and translocates to the nucleus thereby promoting transcription of its target genes (Figure 2)(Barnes and Karin, 1997; Guijarro and Egido, 2001).

Activation of NF-κB upregulates chemoattractant proteins and adhesion molecules allowing monocytes to migrate into tissues further contributing to inflammation (Collins et al, 1993; Baeuerle and Henkel, 1994). Clinical and experimental data confirm the presence of activated NF-κB in a variety of chronic inflammatory diseases such as rheumatoid arthritis, asthma and inflammatory bowel disease (Barnes and Karin, 1997). In the progression of renal disease NF-κB regulates genes such as MCP-1 and RANTES (Guijarro and Egido, 2001; Wang et al, 1999; Zoja et al, 1998). NF-κB does not activate TGF-β1, but it does regulate transglutaminase which is the enzyme responsible for promotion of this growth factor (Mirza et al, 1997). In proteinuric states high albumin concentrations may induce NF-κB activation and tubular injury (Zoja et al, 1998; Wang et al, 1999) which is common to progressive renal disease.
Figure 2. Schematic Diagram of NF-κB activation. Activation of NF-κB involves the phosphorylation of IκB specific IκB kinases. Free NF-κB enters the nucleus, binds to κB in the promoter regions of genes for inflammatory proteins such as chemokines, proinflammatory cytokines, enzymes and adhesion molecules (Based on facts from Barnes & Karin 1997; Mirza et al, 1997).
1.11.2 Transforming Growth Factor-Beta 1

Increased expression of the profibrotic cytokine TGF-β1 is a feature common to many nephropathies that contributes to matrix protein overproduction and type IV collagen expression (Border and Ruoslahti, 1992; Sharma et al, 1997). In early glomerulosclerosis, TGF-β1 influences mesangial cells to revert back to mesangioblasts which can lead to excessive production of ECM and mesangial expansion (El Nahas, 2003). Among growth factors, TGF-β1 is believed to be the most fibrogenic, and can increase transformation of fibroblasts to myofibroblasts, enhance matrix producing genes, and inhibit enzymes responsible for ECM degradation (Basile, 1999; Eddy, 2000).

1.11.3 Monocyte Chemoattractant Protein-1

When locally produced in the kidney MCP-1 can advance tubulointerstitial fibrosis (Viedt and Orth, 2002). In humans and animals with renal disease there are consistent urinary increases in MCP-1 concentration in conjunction with increased urinary albumin/protein excretion and renal damage (Rovin et al, 1996; Wada et al, 1996; Banba et al, 2000; Stephan et al, 2002). In cell cultures of rat proximal tubules exposed to high concentrations of either albumin or transferrin, MCP-1 expression is rapidly induced (Ueda et al, 1994; Wang et al, 1997). Accumulation of MCP-1 into the renal interstitium could be an initial trigger of tubulointerstitial inflammation and fibrosis or a major contributor. When present at this location MCP-1 can activate monocytes and macrophages that increase TGF-β secretion and interstitial fibrogenesis (Wang and Hirschberg, 2000).
1.11.4 Regulated upon Activation Normal T-cell Expressed and Secreted

RANTES is NF-κB dependent (Nelson et al, 1993) and has potent chemotactic activities for macrophages, granulocytes and T lymphocytes (Schall et al, 1990). In mice with crescentic glomerulonephritis and markedly proteinuric, RANTES was up-regulated in the kidney (Lloyd et al, 1997). Zoja et al (1998) showed that in vitro RANTES secretion stimulated by protein overload enters the basolateral compartment of cells. If this was to occur in vivo, RANTES could accumulate in the interstitial space alongside MCP-1 in response to protein overload caused by increased intakes. These chemokines can form concentration gradients which permit the migration of circulating monocytes into the interstitium (Hirschberg and Wang, 2000). Therefore, it is likely that RANTES may very well contribute to inflammatory cell recruitment and subsequent tubulointerstitial fibrosis.

1.11.5 Endothelin-1

The kidney is an important producer of ET-1 and is the target organ of this potent vasoconstrictor (Kohan, 1993). Increased urinary excretion of ET-1 has been observed in experimental animals and humans with glomerular disease and proteinuria (Roccatello et al, 1994; Vlachojannis et al, 1997). The stimulation of tubular epithelial cells with filtered protein triggers transcription of vasoactive and chemokines such as ET-1 and MCP-1 (Healy and Brady, 1998). In the rat 5/6 nephrectomy model of chronic renal failure, increased urinary ET-1 excretion has been observed alongside proteinuria (Bruzzi et al, 1997). It has been shown that increased urinary ET-1 excretion is correlated with the degree of renal insufficiency as measured by serum creatinine and proteinuria, as well as glomerulosclerosis (Larivière et al, 1997; Brooks et al, 1991).
Plasma ET-1 is short lived in the bloodstream and is quickly removed from circulation mainly by the lung and the kidney (de Nucci et al, 1988). Plasma ET-1 concentration may be normal during renal failure, but can be clearly elevated in end-stage renal disease (Lebel et al, 1994; Koyama et al, 1989).

1.12 Hypothesis and Objectives

To the author’s knowledge, most of what is known regarding HP intakes and the progression of renal insufficiency comes from rat studies where there was either a reduction in renal mass or underlying kidney disease. In the short- and long-term studies with healthy rats, isolated protein sources are used to increase protein intakes based on diet weight. Currently, the results are conflicting with regards to renal injury and further clarification of whether HP intakes are harmful to the kidney is needed.

Hypothesis

The hypothesis for this study is that a long-term protein intake at the upper end of the AMDR recommendations (35%) is safe to renal health in the female Sprague-Dawley rat. To test this hypothesis, 79 female normal healthy Sprague-Dawley rats were offered nutritionally and energy balanced diets that contained either 15 or 35% of energy as protein from plant and animal sources.

Objectives

The primary objective of this study was to determine the long-term safety of a diet containing 35% of energy as protein and possible effects on kidney function, pathology, and inflammation. Secondary objectives were to identify potential early histological and early inflammatory markers associated with fibrosis and scarring common to progressive renal disease.
2. METHODS AND MATERIALS

2.1 Experimental Design

Female Sprague-Dawley rats, 10 weeks of age were purchased from Charles River, Saint-Constant, Quebec. Normal rats were used since the primary objective of this study is to determine the safety of the new AMDR protein recommendations with respect to the general healthy Canadian population. Females were selected since this segment of the population is often the target of weight loss strategies. The Sprague-Dawley rat has been previously used in a long-term study involving protein and the effects on the kidney (Bertani et al, 1989). An animal model such as the rat affords the opportunity to examine the long-term effects due to the shorter lifespan. As a result, dietary intervention and its effects on renal function can be evaluated.

The study involved 79 Sprague-Dawley rats, 38 of which were offered a diet containing 15% of energy as protein (normal protein) and 41 were offered 35% of energy as protein (HP). The percentage of protein refers to the amount of protein in energy from daily calories. These levels were selected since 15% represents the average protein intake in the Canadian diet while 35% represents the upper end of the AMDR (IOM, 2002). One quarter of the animals were terminated at each of 4, 8, 12, and 17 months with an \( n = 8-11 \) obtained for each diet group at each time point.

Diets were formulated to be isocaloric to ensure that the nutrient to energy ratio was the same, with the exception of protein. Please refer to Table 2 for the macro- and micronutrient dietary composition. The protein components came from animal and plant protein to emulate the general protein sources found in the human diet. Animal protein sources were pork meal (Rothsay, Winnipeg, MB), poultry meal (Rothsay, Winnipeg,
<table>
<thead>
<tr>
<th></th>
<th>15% ME as CP¹</th>
<th>35% ME as CP¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/100 g diet</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>13.04</td>
<td>31.15</td>
</tr>
<tr>
<td>Fat</td>
<td>4.00</td>
<td>4.00</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>82.96</td>
<td>64.85</td>
</tr>
<tr>
<td>ME</td>
<td>3476</td>
<td>3560</td>
</tr>
<tr>
<td>Animal/Plant Protein Ratio</td>
<td>2</td>
<td>6.25</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.545</td>
<td>0.545</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.425</td>
<td>0.425</td>
</tr>
<tr>
<td>Lactose</td>
<td>7.25</td>
<td>7.25</td>
</tr>
<tr>
<td>Potassium</td>
<td>0.70</td>
<td>0.72</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.45</td>
<td>0.45</td>
</tr>
</tbody>
</table>

ME = metabolizable energy; CP = crude protein.

¹Expressed as % of ME provided as CP
MB), technical egg albumen (Inovatech, Abbotsford, BC) and skim milk powder (Dairyland, Red Deer, AB). The added protein in the HP diet (35%) came from isonitrogenous amounts of egg albumen and skim milk powder. These two animal sources are common ways of increasing protein intake within the human population. The plant protein sources used included wheat and barley from a commercial source (The Puratone Corporation, Niverville, Manitoba).

The diet formulations kept other nutrients similar, and included cornstarch (Casco Inc., Etobicoke, ON), sucrose (Upper Canada Malt, Burlington, ON) lactose (Davisco, Eden Prairie, MN), lard (Canbra Foods, Lethbridge, AB), canola oil (Canbra Foods, Lethbridge, AB), vitamins (AIN93-VX) and minerals (Ca, P, Mg Deficient)(Harlan Teklad, Madison, WI). Please refer to Table 3 for a complete list of ingredients and composition of the diets. Table 4 provides the essential amino acid composition of both diets. The amino acid levels were calculated from the protein feed ingredients used in the study (Rothsay, 2007a; Rothsay, 2007b, National Research Council, 1998; American Egg Board, 2007). Both diets met the essential amino acid requirements for maintenance of rats with the exception of isoleucine in the normal protein (NP) diet (National Research Council, 1995). The requirement for maintenance of isoleucine is 3.1 g/kg of diet, the NP provided 2.49 g/kg (National Research Council, 1995). Diets were prepared weekly by pre-mixing the dry ingredients and adding the fat sources prior to mixing and were stored at -20°C.

Rats were housed in pairs in solid bottom plastic cages with wood shavings and given wooden blocks (to prevent malocclusion), plastic tubes made from the same plastic
Table 3 Composition of normal protein (15% of energy) and high protein (35% of energy) diets.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Normal (15%)</th>
<th>High (35%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>246</td>
<td>246</td>
</tr>
<tr>
<td>Barley</td>
<td>61.50</td>
<td>61.50</td>
</tr>
<tr>
<td>Low Ash Poultry Meal</td>
<td>31.50</td>
<td>31.50</td>
</tr>
<tr>
<td>Pork Meal</td>
<td>22.50</td>
<td>22.50</td>
</tr>
<tr>
<td>Egg Albumen</td>
<td>35.13</td>
<td>247.81</td>
</tr>
<tr>
<td>Skim Milk Powder</td>
<td>78</td>
<td>140</td>
</tr>
<tr>
<td>Sucrose</td>
<td>165.85</td>
<td>83.13</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>262.42</td>
<td>131.53</td>
</tr>
<tr>
<td>Mineral Mix</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Sodium Phosphate Monobasic</td>
<td>0</td>
<td>0.65</td>
</tr>
<tr>
<td>Dicalcium Phosphate</td>
<td>7.41</td>
<td>0.48</td>
</tr>
<tr>
<td>Potassium Chloride</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>6.30</td>
<td>0</td>
</tr>
<tr>
<td>Lactose</td>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td>Vitamin Mix</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Lard</td>
<td>11.29</td>
<td>0</td>
</tr>
<tr>
<td>Canola</td>
<td>11.90</td>
<td>11.90</td>
</tr>
</tbody>
</table>
Table 4 Essential amino acid composition of normal protein (15% of energy) and high protein (35% of energy) diets.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Normal (15%)</th>
<th>High (35%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/kg diet</td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>3.41</td>
<td>4.68</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.35</td>
<td>4.85</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.49</td>
<td>9.93</td>
</tr>
<tr>
<td>Leucine</td>
<td>4.58</td>
<td>17.11</td>
</tr>
<tr>
<td>Lysine</td>
<td>3.62</td>
<td>13.65</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.52</td>
<td>6.49</td>
</tr>
<tr>
<td>Cystine</td>
<td>1.06</td>
<td>4.66</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.69</td>
<td>11.25</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.1</td>
<td>7.96</td>
</tr>
<tr>
<td>Threonine</td>
<td>2.42</td>
<td>4.8</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.72</td>
<td>3.11</td>
</tr>
<tr>
<td>Valine</td>
<td>3.31</td>
<td>12.94</td>
</tr>
</tbody>
</table>
as the cages (to prevent barbering) and a piece of paper towel. These items also provided environmental enrichment. The animals were offered the diets *ad libitum*, 3 times per week in ceramic puppy food dishes (Monday, Wednesday and Friday). Free access to water from the same source (tap water) was provided through glass water bottles which were changed daily. Cages were changed twice per week with food intake being monitored weekly by food disappearance. The amount of food provided was recorded Friday morning and the remaining food was weighed Monday morning. To calculate net feed intake the food that remained was subtracted from the initial weight. Cage intake per day was calculated by dividing by 3 (days), and then divided by 2 (number of rats per cage) to estimate average daily intake per rat. During the treatment period, rats were kept in a controlled environment in which the temperature was maintained at 21-23°C with 55% relative humidity and a 14 hr light, 10 hr dark cycle. Animals were weighed every two weeks from baseline and randomized to diets and termination time points prior to the start of the study.

Urine was collected at 4, 8, and 12 months from 10 HP and 10 NP rats. To achieve this, animals were individually housed in polycarbonate metabolic cages (Nalgene, Fisher Scientific, Fair Lawn, New Jersey) for 6 days. Saphenous blood was collected as previously described by Hem et al (1998) prior to placing the animal in the metabolic cages. The blood was collected in 400 µl BD microtainer serum separator tubes (Becton Dickinson, Franklin Lakes, NJ) to measure serum levels of calcium and phosphorous. Due to the size and weight of the animals at 17 months, urine was not collected with this method.
Following a two-day adaptation period, a 24-hr urine collection was measured in a graduated cylinder (ml), aliquoted into a 15 ml Fisherbrand sterile centrifuge tube, placed on ice and centrifuged at 2400 RPM for 10 minutes at 4°C (Beckman TJ-6 Centrifuge, Palo Alto, CA) to remove any particulate matter. From here, 1 ml was aliquoted into 3 separate 1.5 ml Eppendorf tubes and 1 ml into 2 separate 1.5 ml Eppendorf pyrogen/endotoxin free tubes (5 ml total). The remainder was stored in 6 ml scintillation vials. The urine was frozen at -80°C for analysis of protein, creatinine, and TGF-β1. Excess 24-hr urine was collected on collection days 2 and 3 and aliquoted into 6 ml scintillation vials and frozen at -80°C.

For the 17 month animals, urine was collected from the bladder using a syringe at termination, and frozen at -80°C for analysis of protein and creatinine.

Body composition was measured at 4, 8, 12, and 17 months with a dual energy x-ray absorptiometer (DXA; small animal software; 4500A; Hologic, Inc., Bedford, MA). DXA has been confirmed to be an appropriate method to evaluate body composition in the adult rat (Bertin et al, 1998). Single scans were completed to determine whole body lean mass as well as whole body fat mass. One animal from each diet was scanned in triplicate to measure precision error (co-efficient of variation: CV %) at each time point. Animals were sedated with isoflurine prior to the scan and remained sedated throughout the duration of the scan. All scans were performed with the rat in the anterior-posterior position with limbs extended.

2.2 Measurement of Renal Function

2.2.1 Blood Collection

Animals were injected with Ketamine/Rompun (0.09 ml/100 g body weight) and
exsanguinated via a cardiac puncture with the blood collected placed into 4.0 ml BD Lavender Top Vacutainer plastic plasma tubes coated with 7.2 mg K\textsubscript{2}EDTA (Becton Dickinson, Franklin Lakes, NJ) and 4 ml BD Red Top Vacutainer serum collection tubes then placed on ice (Becton Dickinson, Franklin Lakes, NJ). The BD Vacutainer tubes were centrifuged at 2400 RPM (1200 g) for 15 minutes at 4\textdegree C (Beckman TJ-6 Centrifuge, Palo Alto, CA). Serum was aliquoted into 2-500 \textmu L Eppendorf tubes and the remainder stored in 1-1.5 ml Pyrogen/Endotoxin Free Eppendorf tube. Plasma was aliquoted into 1-500 \textmu L Eppendorf tube and 2-1.5 ml Eppendorf tubes. One of the 1.5 ml Eppendorf’s (plasma) and the renal blood (1.5 ml tube) was centrifuged at 9600 rpm (9500 g) for 10 mins at 4\textdegree C (Eppendorf AG, Hamburg, Germany, model No. 5417C). This second centrifugation of the plasma was to remove any additional platelets. This plasma was aliquoted into 2-500 \textmu L Eppendorf tubes for TGF-\textbeta\textsubscript{1} measurement. The renal plasma was aliquoted into a new 1.5 ml Eppendorf tube. All plasma and serum collected were frozen at -80\textdegree C for future analysis of creatinine clearance.

Urine collected from the rats in metabolic cages was used to assess renal function via protein and creatinine assays. Creatinine is synthesized from free creatine in muscle and the amount of creatinine produced is proportional to muscle mass. Muscle mass or lean body weight can increase in rats when dietary protein is increased from 10 to 20-25\% (Thonney and Ross, 1987). An increase in lean muscle mass is associated with increased serum levels of creatinine. In the absence of renal disease, the excretion rate of creatinine is relatively constant. Therefore, measurement of urinary creatinine, serum creatinine and creatinine clearance are useful markers for kidney function.
2.2.2 Microassay for Total Protein Determination

Urinary and kidney protein concentrations were determined by protein assay using the Bradford method (Bradford, 1976). A Costar 96-well microplate (Corning Incorporation, Corning, New York), was used and labeled as blank, standard or sample. For urine, standard concentrations of 0.05, 0.1, 0.2, 0.3, and 0.5 mg/ml were made using Bovine Serum Albumin (Sigma, St. Louis, Missouri). Ten μL of blank (deionized water), standard, and 2-30X diluted (with deionized water) urine or kidney samples were added to wells in duplicate. Two hundred μL of room temperature Bradford Reagent (Sigma, St. Louis, Missouri) was added to each well and mixed on an orbital shaker (Fisher Scientific, Fair Lawn, New Jersey, Model No. 361) for approximately 15 minutes until there is no precipitate. The plate was read at 595 nm using a SpectroMax Microtiter Plate Reader (Molecular Devices Corporation, Sunnyvale, California). The software included with this plate reader plots the standard curve and calculates the unknowns from the line of the standard curve.

Urine protein was expressed per mg of urine creatinine because the size of the animals at 17 months prevented the use of metabolic cages. Urinary protein was expressed in mg per 24 hours for 4, 8, and 12 months, based on 24 hour urine collection from the metabolic cages.

2.2.3 Microassay for Total Creatinine

Urinary creatinine concentrations (mg/dL) were determined by an adapted creatinine assay (Heinegård and Tiderstrom, 1973). This assay is based on the formation of a yellow/orange colour when creatinine reacts with alkaline picrate. Since this reaction is not specific and a number of proteins in body fluids can interfere, an acid reagent is
added. This ensures that the creatinine-picrate colour fades faster than the colour produced by interfering factors. The difference in colour intensity measured at 500 nm before acidification and after acidification is proportional to the creatinine concentration in the sample.

A Costar 96-well microplate (Corning Incorporation, Corning, New York), was used and labeled as blank, standard or sample. Standard concentrations of 0, 1, 3, 6, 8, and 10 mg/dL were made using a Creatinine Standard Set (Sigma-Aldrich, St. Louis, Missouri, C3613). Twenty μL of blank (deionized water), standard and 20X diluted urine (with deionized water) were added to the wells in duplicate. Two hundred μL of Picric solution (2 parts 0.05 M Sodium Phosphate and 0.05 M Sodium Borate: 2 parts 4% aqueous SDS: 1 part 1.3% Picric Acid) were added to each well and mixed thoroughly on an orbital shaker (Fisher Scientific, Fair Lawn, New Jersey, Model No. 361) at room temperature for 45 minutes. The plate was read at 500 nm using a SpectroMax Microtiter Plate Reader (Molecular Devices Corporation, Sunnyvale, California). Twenty μL of 15% Acetic Acid solution was added to each well and mixed thoroughly and allowed to stand for 5 minutes at room temperature. A second absorbency reading was taken at 500 nm. Creatinine concentration of the unknown samples was calculated using the following equations:

\[
\text{Creatinine Concentration (mg/dL)} = \frac{\text{initial } A \text{ of unknown sample} - \text{final } A \text{ of unknown sample}}{\text{initial } A \text{ of 3 mg/dL standard} - \text{final } A \text{ of 3 mg/dL standard}} \times \frac{3 \text{ mg/dL} \times \text{ DILUTION FACTOR}}{X \text{ mg/dL} \times \text{ Dilution Factor}}
\]

where, \( A = \) absorbance and \(*\) is the concentration of the creatinine standard.

The value of creatinine concentration was multiplied by the dilution factor (20)
before being converted into milligrams of creatinine in a 24 hr urine sample, which was calculated using the following equation:

**Creatinine Excretion (mg/24 hr) =**

\[
\text{Creatinine Concentration (mg/dL) } \times \text{ dL of urine collected in 24 hrs}
\]

2.2.4 Serum Creatinine

The same procedure was used to measure serum creatinine (mg/dL). However, because serum creatinine concentration is lower, standard concentrations of 0.25, 0.5, 1, 3, 5, 6, 8 and 10 were used to provide a similar concentration to the serum creatinine concentrations of the unknown samples. Creatinine clearance was calculated using the following equation:

**Creatinine Clearance (ml/min) =**

\[
\frac{\text{urine creatinine (mg/dL) } \times \text{ urine volume (dL/24 hr)}}{\text{serum creatinine (mg/dL)}}
\]

where, 24 hr was expressed in minutes.

2.3 Histology

At termination the right and left kidneys were removed, with the right kidney flash frozen immediately in liquid nitrogen and frozen at -80°C for future analyses. The left kidney was sectioned transversely and immediately fixed in 10% buffered formalin. The remainder of the left kidney was flash frozen with liquid nitrogen and frozen at -80°C for future analysis of renal inflammatory markers. The kidneys in formalin were left overnight at room temperature and transferred to 0.01 M Phosphate Buffered Saline (PBS) and refrigerated at 4°C until sectioning. The samples were re-fixed in 10%
formalin prior to embedding in paraffin and sectioned at 5 microns with a Microtome (American Optical 820, Southbridge, Massachusetts).

2.3.1 Measurement of Mean Glomerular Volume

Sections for glomerular size were placed in xylene to remove paraffin before being stained with Periodic Acid Schiff (PAS). Sections were first brought to water, and then a 1% aqueous solution of periodic acid was applied for 20 minutes. The sections were washed in water to remove any traces of the periodic acid then the Schiff's reagent was applied for 15 to 30 minutes. The Schiff's reagent was rinsed off with water for 5 minutes, and then stained in Mayer’s Hematoxylin for 3 minutes. The sections were washed for 5 minutes under water. The sections were dehydrated, cleared, and mounted on slides (Kiernan, 1999). PAS stains basement membranes pink and more deeply than Haematoxylin and Eosin to further assist with membrane outlining for glomerular diameter measurement.

To determine the mean glomerular volume (MGV) 30 randomly chosen glomeruli per kidney were measured by a blinded investigator using light microscopy on an Olympus BX60 microscope using a 20X objective. Images were captured by a Junior Spot CCD high-resolution camera (Diagnostic Instruments Inc, Sterling Heights, Michigan) connected to the microscope and projected onto a Sony 19 inch monitor with the use of Spot Advanced software version 3.0.1 (Diagnostic Instruments Inc, Sterling Heights, Michigan). To measure the diameter of each glomerulus, Image Pro Plus software version 6.0 (Media Cybernetics, Del Mar, California) was used. The diameter of each glomerulus was measured at the widest aspect of the aforementioned imaging system on a 20X calibrated grid with each diameter measurement (μm) divided by two to
obtain the radius of each glomerulus. Mean glomerular area (MGA) was calculated using the following formula:

\[ MGA = \pi r^2 \]

**From this, mean glomerular volume (MGV) was calculated as:**

\[ MGV = 1.25 \times (MGA)^{3/2} \]

where, 1.25 is derived from \( \beta/K \), where \( \beta \) equals 1.38 (pertains to spheres) and \( K \) equals 1.10 (a distribution coefficient) (Hirose et al, 1982). The MGV will help determine if the upper level of protein recommendations alters glomeruli size. This is an important step since this is one of the first events to occur in progressive renal disease.

### 2.3.2 Measurement of Glomerulosclerosis

Sections for the quantitative analysis of glomerulosclerosis were stained with Puchtler’s Picro-sirius red technique for collagen. Sections were saturated in aqueous picric acid (14 g picric acid and 200 ml of water) and left to stand overnight. A working Picric Sirius Red solution composed of 1% aqueous Sirius Red F3BA (1 g Sirius Red in 100 ml of water) was mixed with saturated picric acid (20 ml of 1% Sirius Red and 180 ml of saturated picric acid). The sections were then stained in 0.1% Sirius Red F3BA/aqueous picric acid solution for 30 minutes, washed with water, dehydrated in alcohol, and mounted on slides (Puchtler et al, 1973; Kiernan, 1999).

Sirius red stains collagen red, increases the natural birefringence, and stains the cytoplasm yellow. In ordinary bright-field microscopy the intensity of the red colour can then be measured by microdensity to provide an estimate of collagen content in various areas of the tissue (Kratky et al, 1996). According to Junqueira et al (1979), the birefringence is highly specific to collagen which accumulates during renal fibrosis.
To determine glomerusclerosis score, 25 randomly chosen glomeruli per kidney were measured by a blinded investigator with light microscopy on an Olympus BX60 microscope using a 40X objective. Images were captured by a Junior Spot CCD high-resolution camera (Diagnostic Instruments Inc, Sterling Heights, Michigan) connected to the microscope and projected onto a Sony 19 inch monitor with the use of Spot Advanced software version 3.0.1 (Diagnostic Instruments Inc, Sterling Heights, Michigan). Glomerulosclerosis was assessed using hue and saturation densitometry analysis with Image Pro Plus software version 6.0 (Media Cybernetics, Del Mar, California). Blood vessels were used as the reference for collagen deposition and fibrosis.

Glomerulosclerosis was measured by determining total fibrosis of each image and extracting the area outside of the glomerulus which included peritubular and interstitial fibrosis, and blood vessels. The mean proportion of pixels (density) that represented the glomerulosclerosis positive area was then divided by the number of pixels per image (390,500) to generate an objective score of glomerulosclerosis.

2.3.3 Measurement of Tubulointerstitial Fibrosis

Sections for the quantitative analysis of tubulointersitial fibrosis was determined with Puchtler’s Picro-sirius red technique for collagen as per the method used for glomerulosclerosis.

To determine tubulointerstitial fibrosis score, 25 randomly chosen images from the renal cortex were measured by a blinded investigator with light microscopy on an Olympus BX60 microscope using a 10X objective. Images included glomeruli and tubules and were captured by a Junior Spot CCD high-resolution camera (Diagnostic Instruments Inc, Sterling Heights, Michigan) connected to the microscope and projected...
onto a Sony 19 inch monitor with the use of Spot Advanced software version 3.0.1 (Diagnostic Instruments Inc, Sterling Heights, Michigan). Tubulointerstitial fibrosis was assessed using hue and saturation densitometry analysis with Image Pro Plus software version 6.0 (Media Cybernetics, Del Mar, California). Blood vessels were used as the reference for collagen deposition and fibrosis.

Tubulointerstitial fibrosis was measured by determining total fibrosis of each image. The mean proportion of pixels (density) that represented the tubulointerstitial fibrosis positive area (total fibrosis) was then divided by the number of pixels per image (390,500) to generate an objective score of tubulointerstitial fibrosis.

2.4 Measurement of Early Markers of Kidney Disease and Progression

2.4.1 Lyophilization of Kidneys

Each half of the left kidney was lyophilized in preparation for the enzyme-linked immunosorbent assay (ELISA) kits for early inflammatory markers of renal disease and its progression. Frozen kidneys were weighed initially and placed in pre-weighed 15 ml Fisherbrand sterile centrifuge tube test tubes whose lids had holes pierced on the top, immersed in liquid nitrogen and then placed onto a freeze drying apparatus (Virtis, Model No 10-145MR-BA, Gardiner, New York). Tissue samples were dried until two consecutive equal weights were obtained. Dried kidneys were pulverized in the test tube using a metal spatula, and the lid replaced with one without holes and stored at -80°C.

2.4.2 Homogenization of Kidneys

Lyopholized kidney tissue was homogenized using a modification of the method as per Cuozzo et al (2002). A pre-weighed representative sample of lyophilized left kidney tissue (30 mg) was homogenized on ice in 100 volumes of ice-cold particulate
homogenization buffer for a total of 60 seconds using a Polytron homogenizer (Brinkmann Instruments, Mississauga, Ontario). The buffer contained 50 mM Tris-HCl (pH 7.2); 250 mM sucrose; 2 mM ethylene-diamine-tetraacetic acid (pH 7.6); 1 mM ethylene glycol-bis (β-aminoethyle ether) N,N,N’,N’-tetracetic acid (pH 7.5); 50 μM NaF; 0.5% Triton X-100; 100 μM sodium orthovanadate; 25 μg/ml aprotinin; 25 μg/ml pepstatin; 25 μg/ml leupeptin; 1 μg/ml soybean trypsin inhibitor; 10 mM β-mercaptoethanol; and 144 μM 4-(2-aminoethyl) benzene sulfonyl fluoride. The homogenate was transferred into 5 ml tubes and centrifuged at 100,000 g for 35 minutes at 4°C using the Beckman L5-50B ultracentrifuge (Mississauga, Ontario). The supernate fraction was collected and stored immediately at -80°C. This supernatant represents the Triton soluble fraction of the intracellular membranes of the renal cells. Whole cell homogenates were used since the location of intracellular inflammatory proteins is uncertain in kidney. The remaining pellet was resuspended in a representative quantity (0.5 ml) of ice-cold particulate homogenization buffer and stored immediately at -80°C.

2.4.3 ELISA Kits

Using kidneys homogenized in particulate homogenization buffer, renal levels of rat TGF-β1, MCP-1, and RANTES were determined using ELISA. The amount of TGF-β1, MCP-1, and RANTES present in kidney was expressed per mg of kidney protein which was determined in the homogenates by using the Bradford protein determination method. TGF-β1, MCP-1, and RANTES was also expressed per mg dry kidney, mg wet kidney, and whole kidney.
2.4.4 TGF-\(\beta_1\)

For TGF-\(\beta_1\), steady-state and ex vivo release was measured in homogenized kidney tissue and centrifuged urine collected from metabolic cages with a TGF-\(\beta_1\) immunoassay solid phase ELISA kit from R & D Systems, Inc. (Minneapolis, Minnesota). This assay is based on the quantitative sandwich enzyme immunoassay technique. In summary, latent TGF-\(\beta_1\) present in the samples is activated to the immunoreactive form and binds to a specific TGF-\(\beta_1\) immobilized monoclonal antibody present in the walls of the 96 well plate. Any unbound substances are washed away and the immobilized TGF-\(\beta_1\) present in the samples is sandwiched by an enzyme-linked polyclonal antibody specific to TGF-\(\beta_1\). Any unbound antibody-enzyme reagent is removed during the second wash and a substrate is added to the wells. The intensity of the colour is then determined spectrophotometrically, using a microplate reader, and is proportional to the amount of TGF-\(\beta_1\) bound in the initial step.

2.4.5 Verification for Kidney Homogenate

To determine if the TGF-\(\beta_1\) immunoassay was compatible with kidney homogenate and no interference was occurring, 2 (one from each diet) samples were spiked. One hundred \(\mu\)L of activated kidney sample was spiked three times with TGF-\(\beta_1\) standard (2000 pg/ml) as follows: 100 \(\mu\)L standard (200 pg), 50 \(\mu\)L standard and 50 \(\mu\)L RD5-26 (1X) diluent (100 pg), and 25 \(\mu\)L standard and 75 \(\mu\)L RD5-26 (1X) diluent (50 pg) to give a final volume of 200 \(\mu\)L for each. Samples were read neat or unspiked to determine the known amount of TGF-\(\beta_1\) in pg/ml and the spiked amount. Spike recovery was calculated by multiplying the known concentration of the neat (unspiked) sample by 0.1 ml to determine the amount of pg in the sample. This value was then added to the
representative spike (in 0.1 ml) to give total pg. To determine the amount of pg/ml, the number of pg of the neat and spiked sample was then multiplied by 5 (since the total volume of the spiked sample was 200 μL). This final value was then divided by the found concentration of the spike and multiplied by 100. Based on recommendations by R & D, recovery should be in the ranges of 80 to 120%. Recovery ranged from 76.2 to 91.3% with an average of ~85%.

2.4.6 TGF-β1 Methodology

A TGF-β1 96-well microplate coated with a TGF-β1 specific monoclonal antibody was used and labeled as blank, standard, or sample. Standard concentrations of 0, 31.2, 62.5, 125, 250, 500, 100, and 2000 pg/ml were made using a recombinant TGF-β1 standard (2000 pg/ml). One hundred μL of homogenized kidney sample or urine was activated with 20 μL of 1N HCl and neutralized with 13 μL of 1.2N NaOH/0.5 M HEPES after a 10 min incubation period. Fifty μL of assay diluent RD1-21 (for cell culture supernates) was added to each well except the blank. Then 50 μL of control (TGF-β1 kit control), standard and activated kidney sample or urine was added to each well in duplicate, tapped for 1 minute to mix, and left on the bench top at room temperature for 2 hours. Each well was aspirated and washed four times with diluted wash buffer using an eight-channel plate washer (Nunc, Roskilde, Denmark). Once dry, 100 μL of TGF-β1 conjugate was added to each well and incubated on the bench top at room temperature for 2 hrs. The washing process was completed a second time and 100 μL of substrate solution was added to each well. The plate was incubated for 30 minutes at room temperature on the bench top and protected from light. One-hundred μL of stop solution was added and the plate was mixed with gentle tapping. The absorbance of each well was read at 450 nm
using a SpectroMax Microtiter Plate Reader (Molecular Devices Corporation, Sunnyvale, California) which was blanked against the plate blank, and a best fit log-log standard curve was plotted with SOFTmax PRO software version 1.20 (Molecular Devices Corporation, Sunnyvale, California). The concentration of TGF-β1 (pg/ml) of the unknown kidney homogenate samples was read from the plotted standard curve and multiplied by the final dilution factor of 1.3. In urine TGF-β1 was not detectable and the sample concentrations from 12 and 17 months were below the plotted standard curve.

2.4.7 MCP-1

For MCP-1, steady-state levels were measured in homogenized kidney tissue with a MCP-1 solid phase ELISA kit from Biosource International (Camarillo, California). To briefly summarize, an antibody specific to rat MCP-1 has been pre-coated on the walls of the 96 well plate. Samples, standards, unknowns and controls are added to the wells along with a second biotinylated anti-MCP-1 antibody. During the first incubation, rat MCP-1 present in the samples binds simultaneously to the capture antibody (immobilized/well walls) on one site and to the solution phase biotinylated antibody on a second site. After the first washing and removal of excess secondary antibody, streptavidin-peroxidase enzyme is added to bind to the biotinylated antibody and completing the four-member sandwich. After a second incubation and washing to remove the entire unbound enzyme, stabilized chromagen (substrate) is added which acts on the bound enzyme to produce colour. The intensity of the colour is then determined spectrophotometrically, using a microplate reader, and is proportional to the concentration of MCP-1 present in the original sample.
2.4.8 Verification for Kidney Homogenate

To determine if the MCP-1 immunoassay was compatible with kidney homogenate and no interference was occurring, 1 sample was spiked. The kidney sample was spiked twice with MCP-1 standard (750 pg/ml) as follows: 150 μL standard and 150 μL sample (112.5 pg); 75 μL standard + 150 μL sample + 75 μL standard diluent (56.25 pg) to give a final volume of 300 μL for each. Samples were read neat or unspiked to determine the known amount of MCP-1 in pg/ml and the spiked amount. Spike recovery was calculated by multiplying the known concentration of the neat (unspiked) sample by 0.15 ml to determine the amount of pg in the sample. This value was then added to the representative spike (in 0.15 ml) to give total pg. To determine the amount of pg/ml, the number of pg of the neat and spiked sample was then multiplied by 3.33. This final value was then divided by the found concentration of the spike and multiplied by 100. Recovery for these 2 spikes was 72.8 (56.25 pg) and 99.1% (112.5 pg).

2.4.9 MCP-1 Methodology

A MCP-1 96-well microplate coated with a specific rat MCP-1 antibody was used and labeled as blank, standard, or sample. Standard concentrations of 0, 11.7, 23.4, 46.9, 93.7, 187, 375, and 750 pg/ml were made using recombinant rat MCP-1 standard. One hundred μL of control and standard was added to the appropriate wells in duplicate, then 50 μL of standard diluent buffer followed by 50 μL of kidney homogenate to the appropriate wells in duplicate. To each well except the chromagen blank, 50 μL biotinylated anti-MCP-1 was added and the side of the plate tapped gently to mix. The plate was covered and left to incubate for 90 minutes at room temperature. Following this the plate was aspirated and washed 4X with the provided wash buffer. One hundred μL
of streptavidin-HRP working solution was added to each well except the blank, covered and incubated at room temperature for 30 minutes. The above wash cycle was repeated and 100 μL of stabilized chromogen was added, covered and incubated in the dark at room temperature for 30 minutes. After this time period 100 μL of stop solution was added and the plate tapped gently to mix. The plate was read at 450 nm using a SpectroMax Microtiter Plate Reader (Molecular Devices Corporation, Sunnyvale, California). The concentration of rat MCP-1 was determined by multiplying the concentrations by the final dilution factor (2X) of the representative sample.

2.4.10 RANTES

For RANTES, steady-state and ex vivo release was measured in homogenized kidney tissue using a RANTES solid phase ELISA kit from Biosource International (Camarillo, California). To briefly summarize, an antibody specific to rat RANTES has been pre-coated on the walls of the 96 well plate. Samples, standards, unknowns and controls are added to the wells and the rat RANTES antigen binds to the captured (immobilized) antibody on one site. After the first wash, a second specific biotinylated anti-RANTES antibody is added. During the second incubation period, this added antibody binds to the immobilized rat RANTES captured during the first incubation period. Upon removal of this second antibody, streptavidin-peroxidase enzyme is added to bind the biotinylated antibody and completing the four-member sandwich. After a third incubation period and washing, stabilized chromagen (substrate [tetramethylbenzidine]) is added which acts on the bound enzyme to produce colour. The intensity of the colour is then determined spectrophotometrically, using a microplate reader, and is proportional to the concentration of RANTES present in the original sample.
2.4.11 Verification for Kidney Homogenate

To determine if the RANTES immunoassay was compatible with kidney homogenate and no interference was occurring, 2 (one from each diet) samples were spiked. Diluted kidney samples were spiked twice with RANTES standard (2500 pg/ml) as follows: 150 μL standard and 150 μL diluted sample (375 pg); 75 μL standard + 150 μL diluted sample + 75 μL standard diluent (187.5 pg) to give a final volume of 300 μL for each. Samples were read neat or unspiked to determine the known amount of RANTES in pg/ml and the spiked amount. Spike recovery was calculated by multiplying the known concentration of the neat (unspiked) sample by 0.15 ml to determine the amount of pg in the sample. This value was then added to the representative spike (in 0.15 ml) to give total pg. To determine the amount of pg/ml, the number of pg of the neat and spiked sample was then multiplied by 3.33. This final value was then divided by the found concentration of the spike and multiplied by 100. Recovery ranged from 84.3 to 100% with an average of ~91%.

2.4.12 RANTES Methodology

A RANTES 96-well microplate coated with a specific rat RANTES antibody was labeled as chromogen blank, standard, or sample. Standard concentrations of 0, 39, 78.1, 156, 312, 625, 1250, and 2500 pg/ml were made using recombinant rat RANTES standard. Kidney homogenate samples were diluted 4000X in two separate dilutions. The first dilution was 200X were 20 μL of kidney homogenate was added to 3980 μL of 0.01 M phosphate buffered saline (PBS) and mixed thoroughly in a 6 ml scintillation vial. The second dilution was 20X and 20 μL of PBS diluted sample (200X) was added to 380 μL
of standard diluent buffer and mixed thoroughly in a 500 μL Eppendorf tube to give a final dilution of 4000X (200 x 20).

One hundred μL of standard, high and low rat RANTES controls (recombinant rat RANTES), and diluted kidney sample (4000X) were added to the appropriate wells in duplicate, and the plate was tapped gently to mix. The plate was then covered and incubated at 37°C for 2 hours in a CO₂ Incubator (Forma Scientific, Marietta, Ohio). Following this the plate was aspirated and washed 4X (allowing the wells to soak for 20 seconds) with the provided wash buffer. One hundred μL of biotinylated anti-RANTES antibody was added to each well except the chromogen blank, covered and incubated at 37°C for 1 hour. The wash cycle was then repeated and 100 μL of streptavidin-HRP working solution was added to each well except the chromogen blank, covered and incubated at room temperature for 30 minutes. Following a third wash cycle (4X), 100 μL of stabilized chromogen was added to each well, covered and incubated in the dark at room temperature for 30 minutes. After this time period 100 μL of stop solution was added and the plate tapped gently to mix. The absorbance of each well was read at 450 nm using a SpectroMax Microtiter Plate Reader (Molecular Devices Corporation, Sunnyvale, California) which was blanked against the chromogen blank, and a best fit log-log standard curve was plotted with SOFTmax PRO software version 1.20 (Molecular Devices Corporation, Sunnyvale, California). The concentration of RANTES (pg/ml) in the unknown kidney homogenate samples was read from the plotted standard curve and multiplied by the final dilution factor of 4000. The amount of RANTES present in kidney was then expressed per mg of kidney protein which was determined in the
homogenates by using the Bradford protein determination method (pg RANTES/mg kidney protein).

2.5 Statistical Analyses

Results were analyzed by 2X4 ANOVA, with diet and time as factors, using SAS software (SAS, Cary, NC). This study was not treated as a repeated measure analysis since the animals at different time points are not the same. The overall effect of differences between diets over the study duration was reported in text. Normality of the data was assessed using the Shapiro-Wilk’s (W) statistic (W >0.05), and the following distribution plots: Box & Whisker, Histogram, and Residuals vs. Predicted. When data did not follow a normal distribution, the data was transformed by either taking the log or arc tangent. Diet and time effects were considered significant at P <0.05. Outliers were excluded if the plotted residuals exceeded the root mean squared error x 3. The MIXED procedure was used when data did not appear normal after transformation to examine heterogeneity. Otherwise the GLM procedure was used. Contrasts were used when a Diet x Time interaction was P<0.1. After the removal of rats with health concerns, the statistical analysis was identical to that outlined above.

The number of animals in each group was determined by the numbers needed to detect differences in renal histology, since these values generally have the greatest variability. In two recent rodent studies by Ogborn and colleagues, the standard deviation of fibrosis data averaged 30% of the mean (Ogborn et al, 2002; Ogborn et al, 2003). In order to detect a 20% difference between means with this level of variance, an n=10 in each group will give a power of 0.8. Published rat studies that determined histological changes with long-term exposure to a HP diet used 10 rats per group (Bertani et al, 1989;
Maeda et al, 1985), further supporting the number of animals per group. Upon removal of rats with health concerns, the number of rats per group ranged from 4-10.

3. RESULTS

3.1 Body Composition and Food Intake

Firstly, rats that consumed HP had body weights that were ~8% lower than NP (406.3 ± 13.13 g versus 440.64 ± 15.94 g, respectively, P = 0.0056, Figure 3). Similar to body weight, percent body fat was ~24% lower in animals on HP compared to the NP diet (25.64 ± 1.6 versus 33.62 ± 1.76, respectively, P <0.0001, Figure 4). Following suit with a reduction in body fat was a significantly higher level of lean body mass by 13% in the HP rats (72.4 ± 1.52 % compared to 63.96 ± 1.62 % in NP, P <0.0001, Figure 5). The Hp diet had significant positive effects on body composition in female rats.

Food intake as measured by weekly food disappearance in the 17 month rats throughout the study did not differ between dietary treatments (Figure 6, P = 0.7858). For this analysis, weekly 3 day feed weights were averaged for 4 month periods.

A summary table of the diet-time point means and SEMs for body composition and food intake is located in the appendix (Appendix Table 8.2).
Figure 3  Body weights of rats offered normal protein (15% of energy) and high protein (35% of energy) for 17 months. Data is presented as mean ± SEM (n = 8-11). 2X4 ANOVA with P <0.05 considered significantly different. Diet, P = 0.0056 and Time, P <0.0001.

Figure 4  Percent body fat of rats offered normal protein (15% of energy) and high protein (35% of energy). Diet, P <0.0001 and Time, P <0.0001.
Figure 5 Percent lean body mass of rats offered normal protein (15% of energy) and high protein (35% of energy). Diet, $P < 0.0001$ and Time, $P < 0.0001$.

Figure 6 Averaged weekly food intakes of rats housed for 17 months offered normal protein (15% of energy) and high protein (35% of energy) ($n = 6-7$). Diet, $P = 0.7858$ and Time, $P < 0.0001$. 
3.2 Kidney Weights

Kidney weights were ~18% higher in rats that were offered the HP diet compared to NP (1.47 ± 0.05 g versus 1.25 ± 0.04 g, respectively, P <0.0001, Figure 7). Since the HP animals weighed less and had increased lean muscle mass, kidney weights were also expressed based on body weight (BW) and lean body weight (LBW). Renal hypertrophy remained with HP feeding with kidney weights that were ~28% higher when expressed per body weight (HP 0.73 ± 0.023 g kidney weight/100g BW, NP 0.57 ± 0.012 kidney weight/100g BW, P <0.0001, Figure 8) and ~13% higher per gram lean body weight compared to the NP diet (HP 1.03 ± 0.036 g kidney weight/100g LBW, NP 0.91 ± 0.023 g kidney weight/100g LBW, P = 0.0023, respectively, Figure 9).

The kidneys of the HP had ~17% higher levels of protein content compared to the NP group (HP 152.88 ± 5.23 mg/kidney versus NP 130.58 ± 4.22 mg/kidney, respectively, P <0.0001, Figure 10). When expressed per gram of kidney, renal protein content of the HP rats was ~11% lower than that of NP kidneys (3.87 ± 0.20 mg/g kidney versus NP 4.34 ± 0.16 mg/g kidney, respectively, P = 0.0128, Figure 11).

The above is summarized based on diet and time point within the appendix and includes the means and SEMs (Appendix Table 8.2).
Figure 7 Kidney weights of rats offered normal protein (15% of energy) and high protein (35% of energy). Diet, P <0.0001 and Time, P <0.0001.

Figure 8 Kidney weights per body weight of rats offered normal protein (15% of energy) and high protein (35% of energy). Diet, P <0.0001 and Time, P = 0.2574.
Figure 9 Kidney weights per lean body weight in rats offered normal protein (15% of energy) and high protein (35% of energy). Diet, P = 0.0023 and Time, P < 0.0001.

Figure 10 Renal protein content per kidney in rats offered normal protein (15% of energy) and high protein (35% of energy). Diet, P < 0.0001 and Time, P < 0.0001.
Figure 11 Renal protein content per gram kidney in rats offered normal protein (15% of energy) and high protein (35% of energy). Diet, $P = 0.0128$ and Time, $P < 0.0001$. 
3.3 Renal Function

Due to the size and weight of the animals at 17 months, rats were not housed in metabolic cages for 24 hour urine collection. Therefore, 24 hour urinary protein excretion and creatinine clearance could not be calculated for this time point.

Urinary protein excretion per 24 hours was ~4.8 times higher in HP compared to NP rats (54.53 ± 21 mg/24 hr versus 10.19 ± 3.4 mg/24 hr, respectively, P <0.0001, Figure 12). Urinary protein per urinary creatinine was used up to the 17 month time point since bladder urine was collected at termination. Once again those on the HP diet had ~3 times higher urinary protein/urinary creatinine than NP (7.21 ± 1.97 versus 2.39 ± 1.07, respectively, P <0.0001, Figure 13).

There was a trend towards an increase in creatinine clearance or hyperfiltration with HP (HP 1.37 ± 0.086 ml/min, NP 1.15 ± 0.093 ml/min, P = 0.055, Figure 14). When creatinine clearance was corrected for body weight, hyperfiltration was ~29% higher in HP rats compared to NP (HP 0.36 ± 0.023 ml/min/100 g BW, NP 0.28 ± 0.023 ml/min/100 g BW, P = 0.0102, Figure 15). However, when creatinine clearance was corrected for lean body weight, no effect of diet was seen (HP 0.48 ± 0.031 ml/min/100 g LBW, NP 0.43 ± 0.032 ml/min/100 g LBW, P = 0.1143, Figure 16).

For further detail regarding renal function parameters and diet-time point means and SEMS, see Appendix Table 8.3.
**Figure 12** Proteinuria in rats offered normal protein (15% of energy) and high protein (35% of energy) at 12 months. Data is presented as mean ± SEM (n = 10). 2X3 ANOVA with P <0.05 considered significantly different. Diet, P <0.0001 and Time, P = 0.0001.

**Figure 13** Urinary protein/urinary creatinine in rats offered normal protein (15% of energy) and high protein (35% of energy) at 17 months (n = 7-10). Diet, P <0.0001 and Time, P <0.0001.
Figure 14 Creatinine clearance of rats offered normal protein (15% of energy) and high protein (35% of energy) at 12 months. Data is presented as mean ± SEM (n = 8-10). 2X3 ANOVA with P < 0.05 considered significantly different. Diet, P = 0.055 and Time, P = 0.1125.

Figure 15 Creatinine clearance per body weight of rats offered 15 normal protein (15% of energy) and high protein (35% of energy). Diet, P = 0.0102 and Time, P = 0.819.
Figure 16 Creatinine clearance per lean body weight of rats offered normal protein (15% of energy) and high protein (35% of energy). Diet, $P = 0.1143$ and Time, $P = 0.5039$. 
3.4 Renal Histology

Alongside increased kidney size of the HP rats was glomeruli hypertrophy. MGV was 22% higher with HP feeding than animals on NP (2.61 ± 0.09 μm$^3 \times 10^6$ versus 2.14 ± 0.08 μm$^3 \times 10^6$, respectively, P = 0.0017, Figures 17-19). Renal and glomerular hypertrophy is indicative of a physiological compensatory mechanism to increased dietary protein. Animals on the HP diet had 33% more glomerulosclerosis when compared to NP (0.0225 ± 0.0014 versus 0.0169 ± 0.0012, respectively, P = 0.0003, Figures 20-22). There was however, an interaction between diet and time at P = 0.0505 for glomerulosclerosis. Through contrasts it was found that glomerulosclerosis occurred by 12 months of age in the HP rats.

When sclerosis of the renal cortex including glomeruli, tubules, and the interstitial area was measured, no effect of diet was seen between groups (HP 0.042 ± 0.003 versus NP 0.040 ± 0.002, P = 0.8487, Figures 23-25).

A summary of renal histology diet-time point means and SEMs can be found within the Appendix (Appendix Table 8.4).
Figure 17 Mean glomerular volume of rats offered normal protein (15% of energy) and high protein (35% of energy). Diet, \( P = 0.0001 \) and Time, \( P = 0.0032 \).
Figure 18 Cross sections of glomeruli in normal protein (NP) and high protein (HP) rats at 4 and 8 months stained with Periodic Acid Schiff (PAS). Arrows point to individual glomeruli.
Figure 19 Cross sections of glomeruli in normal protein (NP) and high protein (HP) rats at 12 and 17 months stained with PAS. Arrows point to individual glomeruli.
Figure 20 Glomerulosclerosis of rats offered normal protein (15% of energy) and high protein (35% of energy). Diet, P = 0.0003, Time, P < 0.0001, and Diet x Time P = 0.0505. Contrasts showed that the change in glomerulosclerosis occurred by 12 months.
Figure 21 Cross sections of glomeruli in normal protein (NP) and high protein (HP) rats at 4 and 8 months stained with Sirius Red.
Figure 22 Cross sections of glomeruli in normal protein (NP) and high protein (HP) rats at 12 and 17 months stained with Sirius Red.
**Figure 23** Tubulointerstitial fibrosis of rats offered normal protein (15% of energy) and high protein (35% of energy). Diet, $P = 0.8487$ and Time, $P < 0.0001$. 
Figure 24 Cross sections of the renal cortex in normal protein (NP) and high protein (HP) rats at 4 and 8 months stained with Sirius Red.
Figure 25 Cross sections of the renal cortex in normal protein (NP) and high protein (HP) rats at 12 and 17 months stained with Sirius Red.
3.5 Inflammatory Proteins

Renal disease progression can be measured by the presence of TGF-β1. There was no significant statistical difference in renal TGF-β1 levels between diets when expressed based on renal protein content (HP 179.39 ± 7.91 pg/mg renal protein versus NP 174.88 ± 7.14 pg/mg renal protein, P = 0.6469, Figure 26).

Increased levels of MCP-1 and RANTES are associated with renal inflammation, kidney disease, and progression. Surprisingly, animals offered HP had ~16% and ~34% lower renal MCP-1 and RANTES levels than NP when expressed based on renal protein (75.73 ± 2.70 pg MCP-1/mg renal protein versus 90.86 ± 2.89 pg MCP-1/mg renal protein, respectively, P <0.0001, Figure 27; 319.97 ± 33.57 ng RANTES/mg renal protein versus 487.26 ± 42 ng RANTES/mg renal protein, respectively, P <0.0001, Figure 28).

Renal levels of TGF-β1, MCP-1, and RANTES were also expressed based on mg of dry, mg wet lyophilized kidney tissue, and per kidney. When TGF-β1 was expressed based on mg dry and mg wet tissue, no significant effect of diet was demonstrated (Appendix section 8.1.1; Appendix Figures 8.1.4 and 8.1.5), but per kidney, TGF-β1 was significantly higher in HP rats (Appendix section 8.1.1; Appendix Figure 8.1.6). As for MCP-1 and RANTES, the diet lowering effect remained in kidneys of the HP animals (Appendix section 8.1.2 and 8.1.3; Appendix Figures 8.1.7 - 8.1.12), but MCP-1 levels per kidney only decreased in HP rats at 1 year. A complete table of diet-time points for TGF-β1, MCP-1, and RANTES levels based on mg renal protein, mg dry and mg wet lyophilized kidney tissue, and kidney is located within the Appendix Table 8.5.
Figure 26 Levels of renal TGF-β₁ per mg protein in rats offered normal protein (15% of energy) and high protein (35% of energy). Diet, P = 0.6469 and Time, P <0.0001.

Figure 27 Levels of renal MCP-1 per mg protein in rats offered normal protein (15% of energy) and high protein (35% of energy) (n =8-10). Diet, P <0.0001 and Time, P <0.0001.
Figure 28 Levels of renal RANTES per mg protein in rats normal protein (15% of energy) and high protein (35% of energy). Diet, P <0.0001 and Time, P <0.0001.
3.6 Animal Health

Nine months into the study four animals developed mammary tumors, 2 from each diet. A fifth animal, a NP rat was discovered with tumors after 10 months of feeding. The tumors were found at an early stage and did not interfere with ambulation, eating, drinking, or were an issue of welfare. Appendix 8.6.2 outlines tumor size and location for these animals.

The 3 NP animals required surgery to meet the 12 month end-point because of tumor size. The tumors in the HP rats were not a concern and were not removed based on the recommendations of veterinary services. At 10 months the tumors were surgically removed and all animals including HP tumor rats were monitored closely by the animal handling staff. For those that had surgery, they returned to their prior healthy weight and made the 12 month termination point. Prior to 1 year of feeding, one NP animal was terminated early due to a prolapsed bladder.

After 15 months a HP animal had to be euthanized due to heart complications as diagnosed post-mortem and not long after a HP rat was operated on to remove a tumor, but eventually had to be euthanized due to the size and location of the tumor on the abdominal wall. By 16 months, 4 NP rats were euthanized due to tumors, 1 of which had complications after surgical removal.

Similar to the NP animals, 4 HP rats developed tumors by 15 months. These tumors were operable and the rats returned to their previous body weights by the final termination point. Two more rats were found to have tumors, but were not operated on and were monitored closely.
These mammary tumors may be due to predisposition in this particular line of Sprague-Dawley rats. The more probable explanation is the fact that these females were not allowed to breed which led to mammary gland complications. Based on the presence of tumors it was decided that the animals would not be fed up to 20 months. As a result, the study was halted at 17 months of feeding and those animals which had tumors or had undergone surgery were terminated. Appendix Tables 8.6.2 and 8.6.7 provides an overview of tumor prevalence at 12 and 17 months.

### 3.6.1 Results after Removal of Problematic Animals

Upon completion, 16 animals had developed tumors (8 NP and 8 HP). Seven animals (3NP and 4 HP) were operated on to remove the tumors, all of which recovered and made their respected end-points. Two animals were euthanized due to a prolapsed bladder and cardiac complications. Five animals were euthanized early because of tumors (4 NP and 1 HP). Seven animals in total were euthanized and were not included in the analyses of this thesis.

A total of 14 rats (8 NP and 6 HP) were removed from the results for a re-analysis of all parameters. Of these 11 rats, 4 NP and 7 HP had developed mammary tumors. In addition to these, 2 NP rats were excluded due to a significant reduction in body weight and 2 HP rats due to a suspected tumor being hematoma and a bleeding anus. The results were statistically re-analyzed without these animals.

The results remained significant, most parameters marginally increased or decreased in significance, including interactions (Appendix Table 8.12). Kidney weights per LBW lost statistical significance (Appendix section 8.8; Appendix Figure 8.8.3; Appendix Table 8.12) whereas creatinine clearance (ml/min) (Appendix section 8.9;
Appendix Figure 8.9.3; Appendix Table 8.12) and MCP-1 levels per kidney increased to statistical significance (Appendix section 8.11; Appendix Figure 8.11.6; Appendix Table 8.12).

4. DISCUSSION

The results of this long-term study demonstrate that chronic HP diets in line with the upper end of the AMDR for dietary protein have negative implications on renal function and glomerular histology in female Sprague-Dawley rats. The current stance of this topic remains debatable within the literature. At the center is a hypothesis over two decades old, largely based on animal studies that states excess protein stresses the kidneys, especially in those previously damaged or diseased. Excessive dietary protein leads to an increase in glomerular pressure and renal hyperfiltration, which cause renal injury and ultimately compromise function and increase the risk for or progression of renal disease (Brenner et al, 1982, Meyer et al, 1991). Bertani et al (1986) further suggested that excess protein filtered through the glomeruli could very well be toxic and initiate further progression. In aging rats whose kidneys are intact, permselective changes occur with time at the glomerulus in response to plasma protein filtration which results in proteinuria, progressive glomerulosclerosis, and eventually renal insufficiency (Anderson and Brenner, 1986; Brenner et al, 1982; Couser and Stilmant, 1975; Elema and Arends, 1975). Therefore, in the presence of HP feeding this age-related damage could be further enhanced via dietary contributions. Although research regarding HP diets and renal safety in healthy individuals has not been thoroughly documented within the literature, the results of this study further support the above hypothesis with regards to normal healthy
female rats. The underlying mechanisms of increased protein intake and its detrimental effects to the kidney remain poorly elucidated.

As an organ of filtration, the kidney can maintain homeostasis when its workload is increased. Kidneys have a predetermined limit of nephrons for functioning that can not increase in number, only size. Early research in rats fed HP diets demonstrated this renal hypertrophy (Osborne et al, 1926; Wilson, 1933). With this increase in work capacity provided by increased dietary protein, elevations in GFR are seen (Brenner et al, 1982; Klahr and Purkerson, 1998; Hostetter et al, 1986; Kaysen et al, 1989). With HP intakes there is an increase in glomerular capillary blood pressure ($P_{GC}$) or hyperfiltration which can be reduced with protein restriction (Anderson and Brenner, 1986). With respect to protein sources, animal protein has the most substantial effect on $P_{GC}$, followed by dairy and plant, respectively (Kontessis et al, 1990; Wiseman et al, 1987; Bilo et al, 1989). In the present study, renal hypertrophy occurred with HP feeding with a trend towards higher GFR as measured by creatinine clearance at 4, 8, and 12 months which became significant when corrected for body weight. When expressed based on lean body weight, creatinine clearance did not differ between the two levels of protein offered. The HP diet consisted of mixed protein sources which may provide some protection against drastic increases in GFR compared to the high intakes of purified protein sources often used in animal studies involving the kidney.

Alterations in GFR and glomerular pressure compromise the permselectivity of the glomerulus. Proteins that are normally too large such as albumin, pass through and contribute to an increase in mesangial matrix and glomerulosclerosis development (Brenner et al, 1982; Couser and Stilmant, 1975). Glomerulosclerosis, common to aging
kidneys and disease contributes to the eventual progression of end-stage renal disease. It is hypothesized that the development of this pathological process can be attributed to dietary protein content, proteinuria, and glomerular hypertrophy (Klahr et al., 1988). In clinical situations and experimental diseases including high dietary protein intakes, nephrectomy, and chemically induced diabetes (Fogo and Ichikawa, 1992; Lafferty and Brenner, 1990; Meyer et al., 1991), glomerular hypertrophy is followed by glomerulosclerosis. In a long-term age dependent glomerular damage study by Baylis (1994), male Munich Wistar rats fed standard rat chow (24% protein) had increased glomerular volumes at 8-12 months of age which preceded renal injury development.

This cascade of events was seen in the present study where proteinuria and urinary protein expressed per urinary creatinine was significantly elevated in HP diets at 12 and 17 months, respectively. In line with excess protein excretion, animals offered HP demonstrated glomeruli hypertrophy with glomerulosclerosis. By 1 year of feeding, kidneys of the HP rats had more glomerulosclerosis than NP with changes in MGV seen as early as 4 months. To compensate for the increased workload of filtration provided by excess dietary proteins the glomeruli of the HP rats increased in volume.

The extent of proteinuria can have detrimental effects on renal function and subsequent progression to renal disease, but this does not necessarily lead to CKD. This was observed in the current study where feeding of a long-term HP diet resulted in minimal change disease with elevated proteinuria and glomerulosclerosis. In heavy proteinuria, mainly albuminuria, tubulointerstitial scarring can be absent (Branten et al., 2001). Tubulointerstitial fibrosis correlates best with loss of renal function and risk of disease progression to kidney failure, not necessarily glomerular damage (Viedt and Orth,
In the current study, early glomerular damage was more apparent than renal function deterioration and end-stage renal disease progression.

The majority of studies that have examined HP diets and renal injury have focused on rat models with reduced nephron mass, which enhances the negative effect of HP on renal function and histology (Provoost, 1989; Kleinknecht, 1979). One such example was by Hostetter et al (1986) who studied the chronic effects of 40% dietary protein on intact, unilaterally, and one-third nephrectomized rats. After 8 months of feeding, rats fed HP had increased urinary protein levels and more sclerotic glomeruli than those on the low protein diet (6%) at all levels of renal mass. The HP diet and in concert development of proteinuria and glomerulosclerosis was related to initial renal mass, and restriction of dietary protein slowed renal injury development.

In the present study, rats with normal healthy kidneys regardless of diet exhibited lower levels of glomerular hypertrophy and glomerulosclerosis compared to what has been reported in rats with reduced nephron mass (Kleinknecht, 1979; Hostetter et al, 1986). The current study is similar to a recent long-term study that examined the effect of a minimum electrolyte diet that contained 50% dietary protein from casein. The normal male Munich-Wistar rats in this long-term study also had renal injury that was comparatively less than animals with reduced nephron mass (Wesson et al, 2007). Although the damage to normal kidneys was less in the females studied in the current study, glomerular damage did occur as demonstrated by increased urinary protein, glomeruli hypertrophy, and glomerulosclerosis. If renal injury was induced or renal mass reduced, it is very likely that the damage would have been more severe.
The histological effects of the HP diet seen in the present study were different than Wesson et al (2007) where there was no change in glomerulosclerosis, but a significant effect of 50% casein on tubulointerstitial fibrosis after 96 weeks. As the male Munich-Wistar rat ages, the genetic susceptibility to glomerulosclerosis and tubulointerstitial injury increases (Grond et al, 1986). Protein intake was higher, composed primarily of casein, and 24 weeks longer in feeding duration. These three factors may attribute to the differences seen in histology between studies.

In a very similar study to the current investigation, male Sprague-Dawley rats with normal kidneys fed 42% protein as energy had significantly worsened proteinuria, tubulointerstitial fibrosis, and focal glomerulosclerosis after 20 months compared to those fed the standard diet (Bertani et al, 1989). Interestingly, rats fed 23% of protein as energy (standard diet) had tubulointerstitial damage and focal glomerulosclerosis which is consistent with laboratory rats freely offered standard casein chow for 2 years (Coleman et al, 1977; Couser and Stilmant, 1975).

The absence of tubulointerstitial fibrosis in the females studied here could be explained by the following. Generally speaking, male rats are more susceptible than females to renal disease progression with advancing age (Gray, 1986) whose decline in renal function is delayed (Wesson, 1969; Gray et al, 1982). Gender differences attributed to the sex hormone androgen may contribute to an increase in glomerular lesion severity in males (Rao, 2002). This theory was demonstrated in a study by Baylis (1994) in which ovariectomized female rats developed glomerular damage with advancing age while in comparison the androgen deficient castrated males were protected from glomerular injury. Also, as with Wistars, Sprague-Dawley rats are highly susceptible to nephropathy
development suggesting genetic predisposition. It is possible specific strains develop
certain renal histological consequences at different time points and with varying degrees
of dietary protein sources and levels. More importantly, glomerulosclerosis often
precedes tubulointerstitial scarring and the associated renal fibrosis (Kriz et al, 1998b)
which could have developed in the current study if feeding had continued longer.

The direct link between glomerulosclerosis and the development of
tubulointerstitial fibrosis however, is unclear. Firstly, repeated glomerular injury could
trigger the glomerulus or tubule cells to release growth factors and chemokines (Border
Secondly, the injured glomerulus could leak proteins that when reabsorbed at the
proximal tubules by endocytosis leads to inflammatory cell formation and growth factor
and chemokine secretion (Remuzzi et al, 1997; Burton and Harris, 1996). Finally, if left
untreated, lesions associated with the development of tubulointerstitial fibrosis may
further contribute to scarring, wasting, and nephron destruction as far back as the initially
injured glomerulus where altered protein filtration occurs (Remuzzi and Bertani, 1997,
Kliem et al, 1996). It is possible for these pathways to work independently, but more
likely they work in conjunction. The initial renal injury, renal mass, gender, or
experimental disease also could affect progression. In the context of the current
experiment, increased protein traffic from a long-term HP diet is the likely culprit. If this
were to persist the associated hyperfiltration, glomerulosclerosis, and proteinuria could
trigger renal inflammatory cues such as the growth factor cytokine TGF-β.

TGF-β forms a high molecular weight complex in serum that cannot normally
pass through the glomerulus, but in a proteinuric state it can become part of the
ultrafiltrate and signal an inflammatory cue in tubular cells (Hirschberg and Wang, 2005; Wang and Hirschberg, 2000). This occurs presumably alongside the glomerular ultrafiltration of the larger molecular weight plasma proteins. Within these cells are TGF-β receptors which can activate cell injury and increase the expression of collagen and fibronectin (Ando et al, 1998; Wang and Hirschberg, 2000). In a similar fashion, the glomerulus may also produce TGF-β which can trigger an inflammatory response in the proximal tubules (Hirschberg and Wang, 2005). Once the inflammatory state is present, activation of tubular cells can increase the migration of chemokines such as MCP-1 and RANTES into the interstitium. The normal kidney expresses low amounts of MCP-1, but in glomerulonephritis, diabetic nephropathy, and partial nephrectomy these levels become increased (Rovin et al, 1996; Tam et al, 1996; Lianos et al, 1994; Lee et al, 1995). In a study that examined renal tubular cells and proteinuric exposure, albumin and transferrin induced MCP-1 production (Wang et al, 1997). As for RANTES, in vitro studies by Zoja et al (1998) have shown that exposure of proximal tubule cell cultures to bovine serum albumin causes concentration and time dependent increases in production. In the tubules of chronic progressive membranous glomerulonephritis patients, overexpression of MCP-1 and RANTES as well as osteopontin was correlated with an accumulation of inflammatory cells in the interstitium (Mezzano et al, 2000). When elevated the role of these chemokines is to attract macrophages, which can increase the secretion of highly fibrogenic TGF-β (Border and Noble, 1994; Roberts et al, 1992; Eddy, 1996).

The release of TGF-β increases matrix production and decreases its degradation, further contributing to sclerosis and renal fibrosis (Klahr et al, 1988; Border and Noble, 1994). At a cellular level, TGF-β1 regulates fibroblast proliferation to myofibroblasts
which produce ECM at levels which lead to an accumulation in the interstitium and fibrosis. In experimental models of glomerulonephritis, glomerulosclerosis, and tubulointerstitial fibrosis overexpression of TGF-β at the gene or protein level was associated with worsened renal pathology (Okuda et al, 1990; Ding et al, 1993; Nakamura et al, 1993; Tamaki et al, 1994; Eddy and Giachelli, 1995; Yamamoto et al, 1994). Contrarily, protein restriction has been shown to reduce TGF-β expression and also delay renal fibrosis in animal models of progressive renal disease. When nephropathy was induced in male Sprague-Dawley rats by Adriamycin fed 5% protein by weight for 8 weeks, there was a reduction in the synthesis and mRNA expression of TGF-β₁ compared to rats fed 20% protein by weight (Nakayama et al, 1996). Nephrotic syndrome induced by puromycin aminonucleoside was attenuated in rats through low protein feeding (8% protein by weight) with decreased renal expression of TGF-β₁ compared to those fed 27% protein by weight (Eddy, 1994). Finally, in comparison to a 25% casein by weight diet fed to Sprague-Dawley males with experimentally induced glomerulonephritis, 7% casein suppressed TGF-β₁ expression and prevented the formation of ECM in the injured glomeruli (Okuda et al, 1991). Further accumulation of inflammatory proteins in the renal interstitium could determine the progression of renal disease to end-stage renal failure.

However, in the present study levels of TGF-β₁ did not differ between diets and as a result chemokine levels did not increase. Conversely, the proteinuric rats in this study had significantly lower levels of MCP-1 and RANTES compared to NP. These lower levels support the absence of tubulointerstitial fibrosis.
The question is whether the HP diet lowered chemokine levels in the kidney, or if the NP animals had increased renal levels of MCP-1 and RANTES. The body composition of the NP animals pointed towards obese animals. Obesity can be defined as a low grade inflammatory reaction and adipocytes increase the expression and secretion of the cytokines MCP-1 and RANTES as body fat rises (Kim et al, 2006; Wu et al, 2007). If blood levels of these chemokines became elevated due to increased body fat, it is possible that they were filtered at the kidneys of NP rats where they could accumulate.

In minimal change disease, the glomerulus remains selective and lower molecular weight proteins and albumin are filtered (Nadasdy et al, 1994). Anything larger than albumin, such as the TGF-β complex is not freely passed. In kidneys of HP rats perhaps permselectivity was not compromised enough to allow the leakage of larger weight proteins and the development of tubulointerstitial fibrosis. The absence of urinary TGF-B1 in both diet groups at 12 and 17 months supports this. Since molecular weights and types of urinary proteins was not measured the above cannot be directly concluded.

When reabsorbed at the epithelium of the tubular cells, proteins and albumin are catabolized via endocytosis and the apical membrane forms lysosomes which digest these molecules into smaller fragments which can be released into the tubular lumen (Burne et al, 1998). The absence of tubulointerstitial damage may have been due to the higher level of lysine in the HP diet. This amino acid is a potent inhibitor of albumin uptake at the luminal membrane of proximal tubules (Tucker et al, 1993). This is accomplished by endocytosis inhibition, altering the negative of charge of albumin to prevent passage, and decreasing tubule brush border height and absorption capacity (Osicka et al, 1999; Tucker et al, 1993). Perhaps the increased content of lysine provided by the extra skim milk
powder and egg albumen in the HP diet exerted a beneficial effect. By increasing exposure to this amino acid, the expression of MCP-1 and RANTES was possibly downregulated compared to NP kidneys.

As for glomerulosclerosis, the positive aspect of lysine may not be apparent until it is filtered at the glomerulus and enters the tubules. Sclerotic glomeruli did appear in this study in correlation with proteinuria, but did not translate to tubulointerstitial damage and loss of renal function. Due to no change in renal levels of TGF-β1 and a decrease in MCP-1 and RANTES, the mechanism behind glomerulosclerosis in the HP rats likely is not due to growth factors or inflammatory cells.

Instead, it is possible that the mechanical process of glomerular hyperfiltration (capillary hypertension) via proteinuria initiated the injury (Klahr et al, 1988). The mesangium of the glomerulus is in direct contact with constituents of plasma and is responsible for keeping the ultrafiltrate free of debris like aggregated proteins. An increased uptake of serum proteins and glomerular capillary wall expansion coupled with aging speeds up the progression of glomerulosclerosis (Daniels and Hostetter, 1990; Grond et al, 1982). The mesangium is also capable of ECM production via interleukin-1 and platelet derived growth factor. Unfortunately, the current study does not provide direct support for the mechanism of mechanical forces inducing sclerosis of the glomeruli.

The present study indicates that long-term HP intakes of mixed sources at the upper end of the AMDR for protein (35%) are associated with minimal change renal disease in healthy female rats. These results suggest hyperfiltration of increased dietary protein which were associated with glomerular damage. The kidney and glomerulus
hypertrophied to compensate for the increased protein load which subsequently led to an increase in proteinuria and the development of glomerulosclerosis. However, tubulointerstitial fibrosis which is correlated to end-stage renal disease was not apparent and was further supported by no change in TGF-β1 and decreased levels of MCP-1 and RANTES. HP feeding of mixed protein sources throughout the rats’ lifespan resulted in glomerular damage in healthy kidneys, but did not progress to renal disease or failure. However, the devastating effect of a HP diet on compromised kidneys has been demonstrated in various models of renal disease.

The implications for humans are simple. The general public is often unaware of compromised renal function and most individuals believe they are healthy even in the presence of stage 1 classification of renal disease. A significant portion of the North American population has at least mild to moderate renal function impairment and these are the individuals at an increased risk to a HP diet. Renal function also declines naturally with age, and as the population ages the dietary effect could become more pronounced. Obesity and the Metabolic Syndrome are on the rise especially at younger ages which puts additional strain on the kidney and enhances the development of glomerulopathy. These individuals may already be consuming large quantities of animal proteins or looking for weight loss strategies that promote HP intakes. Therefore, the current AMDR of 35% appears to be set too high based on the negative implications on healthy kidneys in the present study, and could accelerate renal disease progression in those individuals unaware of their condition.
5. STRENGTHS AND LIMITATIONS

The primary strength of this study was the length and duration involved. As a result, a lifetime of data in a rat model was collected from an age of post-adolescence (sexual maturity) to the elderly. To the author’s knowledge, this is one of very few studies that have examined the long-term implications of a HP diet in the kidneys of normal healthy rats. Furthermore, the lifespan of a laboratory rat is approximately 2 years which permitted the long-term assessment of the renal impact of a HP diet in a reasonable time frame.

A secondary strength was the levels of protein chosen for each diet and the sources. The IOM acknowledges the lack of data at the upper level of 35% daily protein, but this study has demonstrated interesting findings concerning renal safety at this dietary intake in rats. The NP diet was formulated to reflect the average North American intake of daily protein and represents an excellent control. The protein ingredient sources selected to increase the HP diet also mimic common sources to increase intake and humans, skim milk powder (whey) and egg albumen (egg whites).

With respect to histology, the researcher was blinded to avoid any bias, thereby increasing credibility.

A limitation of this study was the animal model chosen; the investigator had no control over the line of Sprague-Dawley rat and was not aware of possible genetic complications such as tumors. Since these females were not allowed to breed, this also increased the likelihood of mammary tumors. The length of the study after 1 year was problematic with respect to tumor occurrence and size limitations for metabolic cages. As
a result, 24 hour urine collection was not possible. This limited a few parameters of renal
function at 17 months such as creatinine clearance and urinary protein excretion.

Limiting the study to females ruled out any possible gender differences and leaves
the question of how male rat’s kidneys could be affected by a HP diet.

For the inflammatory protein markers of kidney disease, the respected companies
had not yet proved the kits for renal levels TGF-β₁, MCP-1, and RANTES kits. Instead,
the author in the laboratory provided verification, but they were not specifically designed
to measure renal levels. This verification however strengthens their credibility.
Secondary, other reliable markers of inflammation such as tumor necrosis factor-1,
vascular endothelial growth factor, interleukin-1, and platelet derived growth factor
would have been useful.
6. DIRECTIONS FOR FUTURE RESEARCH

Even though this long-term study is complete and has shed new light on possible renal implications of a HP intake at the upper rend of the AMDRs in normal female rats, new questions have arisen. The following would be beneficial in the investigation of a HP diet and renal health:

- HP diet effects in males to compare gender effects
- Selection of rat line that has not been in-bred to avoid long-term genetic complications like cancer
- Larger metabolic cages that are comfortable for the animal and allow urine collection at future time points
- Different levels of mixed protein intakes that help determine what level is safe, and at what level negative effects are seen (eg. starting at 20%, 25%, 30%, and finally 35%)
- Decrease carbohydrate, and increase fat in both diets to achieve a balanced diet that parallels that of the AMDRs for humans
- Renal immunohistochemistry of inflammatory markers NF-κB, TGF-β1, MCP-1, RANTES, and ET-1 to further explore production and location within the kidney
- mRNA expression of NF-κB, TGF-β1, MCP-1, RANTES, and ET-1 to determine renal expression of these factors shown to be involved in renal disease
- Examination of other growth factors Insulin Like Growth Factors and Vascular Endothelial Growth Factor which are also associated with renal fibrosis
- Measurement of P_{GC} via micropuncture techniques to determine effects of protein levels on renal hemodynamics
• Analysis of prostaglandins (TxA₂ and PGE₂) which are vaso- constrictors and dilators that influence hemodynamics

• The use of an animal model that resembles humans more closely, such as the pig

• The effects of a HP diet on animal models of obesity and metabolic syndrome such as the Zucker rat because of the link between renal disease progression and obesity
7. REFERENCES


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8. APPENDIX

8.1 Inflammatory Proteins Expressed Based on mg dry, mg wet Kidney Tissue, and per Kidney

8.1.1 TGF-β1

There was no significant difference between dietary treatments when the amount of TGF-β1 was expressed per mg dry kidney (HP 92.55 ± 3.86 pg/mg dry kidney versus NP 89.49 ± 4.14 pg/mg dry kidney, \(P = 0.2973\), Appendix Figure 8.1.4). The same was true when TGF-β1 was expressed per mg wet kidney (HP 19.07 ± 0.84 pg/mg wet kidney versus NP 18.87 ± 0.94 pg/mg wet kidney, \(P = 0.6132\), Appendix Figure 8.1.5) When expressed as total TGF-β1 per kidney, HP animals had levels of TGF-β1 that were ~18 % higher than NP (27333.8 ± 1447.81 pg/kidney versus 23004.14 ± 1183.57 pg/kidney, respectively, \(P = 0.0043\), Appendix Figure 8.1.6).

8.1.2 MCP-1

Animals that consumed HP had ~16% lower levels of MCP-1 than NP per mg dry kidney (38.89 ± 1.19 pg/mg dry kidney versus 46.17 ± 1.55 pg/mg dry kidney, respectively, \(P <0.0001\), Appendix Figure 8.1.7) and ~17 % lower levels of MCP-1 per mg wet kidney (8.00 ± 0.26 pg/mg wet kidney versus 9.68 ± 0.32 pg/mg wet kidney, respectively, \(P <0.0001\), Appendix Figure 8.1.8). There was an interaction of Diet x Time in renal levels of MCP-1 per mg dry tissue (\(P = 0.0884\)). Contrasts showed that at 12 months HP had significantly lower levels of this inflammatory protein. When the data was expressed per kidney, the effect of diet on MCP-1 was absent (HP 11.38 ± 4.67 ng/kidney versus NP 11.93 ± 5.62 ng/kidney, \(P = 0.1433\), Appendix Figure 8.1.9). There was however an interaction of Diet x Time (\(P = 0.0574\)). Contrasts showed that NP animals had significantly higher total renal levels of MCP-1 at 12 months.
8.1.3 RANTES

RANTES in the kidneys of HP animals were ~33% lower than NP per mg dry kidney (323.92 ± 29.16 ng/mg dry kidney versus 486.59 ± 38.35 ng/mg dry kidney, respectively, P <0.0001, Appendix Figure 8.1.10) and ~35% lower levels of RANTES per mg wet kidney (65.85 ± 5.8 ng/mg wet kidney versus 101.22 ± 7.6 ng/mg wet kidney, respectively, P <0.0001, Appendix Figure 8.1.11).

Per kidney, HP rats had ~23 % lower levels of RANTES than NP (98.27 ± 11.29 µg/kidney versus 127.61 ± 11.7 µg/kidney, respectively, P = 0.0019, Appendix Figure 8.1.12). Expressing MCP-1 and RANTES per whole kidney brought the levels down. RANTES remained significantly lower in the kidneys of HP rats.
Appendix Figure 8.1.4 Levels of TGF-β₁ per dry kidney in rats offered normal protein (15% of energy) and high protein (35% of energy). Data is presented as mean ± SEM (n = 8-11). 2X4 ANOVA with P < 0.05 considered significantly different. Diet, P = 0.2973 and Time, P < 0.0001.

Appendix Figure 8.1.5 Levels of TGF-β₁ per wet kidney in rats offered normal protein (15% of energy) and high protein (35% of energy). Diet, P = 0.6132 and Time, P < 0.0001.
Appendix Figure 8.1.6 Levels of TGF-β1 per kidney in rats offered normal protein (15% of energy) and high protein (35% of energy). Diet, $P = 0.0043$ and Time, $P < 0.0001$.

Appendix Figure 8.1.7 Levels of MCP-1 per dry kidney in rats offered normal protein (15% of energy) and high protein (35% of energy) ($n = 8-10$). Diet, $P < 0.0001$, Time, $P < 0.0001$, and Diet x Time, 0.0884. *Contrasts demonstrated that HP animals had lower levels of MCP-1 per dry kidney at 12 months.
Appendix Figure 8.1.8 Levels of MCP-1 per wet kidney in rats offered normal protein (15% of energy) and high protein (35% of energy). Diet, P < 0.0001 and Time, P < 0.0001.

Appendix Figure 8.1.9 Levels of MCP-1 per kidney in rats offered normal protein (15% of energy) and high protein (35% of energy). Diet, P = 0.1433, Time, P < 0.0001, and Diet x Time, P = 0.0574. *Contrasts showed that MCP-1 levels were significantly lower in HP kidneys at 12 months.
Appendix Figure 8.1.10 Levels of RANTES per dry kidney in rats offered normal protein (15% of energy) and high protein (35% of energy) (n = 8-10). Diet, P < 0.0001 and Time, P = 0.0002.

Appendix Figure 8.1.11 Levels of RANTES per wet kidney in rats offered normal protein (15% of energy) and high protein (35% of energy). Diet, P < 0.0001 and Time, P = 0.0025.
Appendix Figure 8.1.12 Levels of RANTES per kidney in rats offered normal protein (15% of energy) and high protein (35% of energy). Diet, $P = 0.0019$ and Time, $P < 0.0001$. 
**Appendix Table 8.2** Body composition, feed intake, and kidney parameters in rats offered normal protein (NP) or high protein (HP) diets.\(^1\)

<table>
<thead>
<tr>
<th>Diet</th>
<th>Time</th>
<th>NP 4</th>
<th>HP 4</th>
<th>NP 8</th>
<th>HP 8</th>
<th>NP 12</th>
<th>HP 12</th>
<th>NP 17</th>
<th>HP 17</th>
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<td></td>
<td>± 11.6</td>
<td>± 10.51</td>
<td>± 19.75</td>
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<td>± 17.99</td>
<td>± 20.41</td>
<td>± 38.2</td>
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<td>± 2.6</td>
<td>± 2.06</td>
<td>± 3</td>
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<td>Percent Body Fat</td>
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<td>± 2.16</td>
<td>± 2.18</td>
<td>± 2.6</td>
<td>± 1.93</td>
<td>± 3.06</td>
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<td>Percent Lean Body Mass</td>
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<td>± 0.4</td>
<td>± 0.48</td>
<td>± 0.57</td>
<td>± 0.6</td>
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<td>Food Intake (g/day)(^2)</td>
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<td>1.161</td>
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<td>1.353</td>
<td>1.556</td>
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</tr>
<tr>
<td>Kidney Weight (g)</td>
<td></td>
<td>0.56</td>
<td>0.69</td>
<td>0.58</td>
<td>0.69</td>
<td>0.56</td>
<td>0.71</td>
<td>0.59</td>
<td>0.84</td>
<td>&lt;0.0001</td>
<td>0.2574</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 0.011</td>
<td>± 0.018</td>
<td>± 0.018</td>
<td>± 0.028</td>
<td>± 0.021</td>
<td>± 0.038</td>
<td>± 0.045</td>
<td>± 0.067</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g Kidney Weight/100 g BW</td>
<td></td>
<td>0.78</td>
<td>0.86</td>
<td>0.88</td>
<td>0.93</td>
<td>0.93</td>
<td>1.05</td>
<td>1.09</td>
<td>1.27</td>
<td>0.0023</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 0.022</td>
<td>± 0.028</td>
<td>± 0.042</td>
<td>± 0.035</td>
<td>± 0.021</td>
<td>± 0.043</td>
<td>± 0.041</td>
<td>± 0.083</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g Kidney Weight/100 g LBW</td>
<td></td>
<td>99.42</td>
<td>122.8</td>
<td>130.69</td>
<td>160.19</td>
<td>149.62</td>
<td>159.39</td>
<td>145.62</td>
<td>167.65</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 3.62</td>
<td>± 5.11</td>
<td>± 3.68</td>
<td>± 5</td>
<td>± 5.7</td>
<td>± 7.32</td>
<td>± 9.03</td>
<td>± 6.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg Protein/Kidney</td>
<td></td>
<td>4.99</td>
<td>4.67</td>
<td>4.92</td>
<td>4.73</td>
<td>4.05</td>
<td>3.60</td>
<td>3.17</td>
<td>2.59</td>
<td>0.0128</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 0.27</td>
<td>± 0.22</td>
<td>± 0.29</td>
<td>± 0.54</td>
<td>± 0.17</td>
<td>± 0.15</td>
<td>± 0.19</td>
<td>± 0.14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Values are the means ± SEM, n=8-11 per diet-time point, NP diet contained 15% and HP diet contained 35% protein as energy.

\(^2\)For food intake, data is expressed as an weekly average of cage mates/day per termination point, n=6-7.
Appendix Table 8.3 Renal function as measured by proteinuria, urinary protein per urinary creatinine, and creatinine clearances in rats offered normal protein (NP) or high protein (HP) diets.1

<table>
<thead>
<tr>
<th>Diet</th>
<th>Time</th>
<th>NP 4</th>
<th>HP 4</th>
<th>NP 8</th>
<th>HP 8</th>
<th>NP 12</th>
<th>HP 12</th>
<th>NP 17</th>
<th>HP 17</th>
<th>Diet P</th>
<th>Time P</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td></td>
<td>2.78 ± 0.21</td>
<td>12.77 ± 6.97</td>
<td>15.09 ± 8.29</td>
<td>32.77 ± 8.94</td>
<td>14.19 ± 4.31</td>
<td>109.13 ± 55.15</td>
<td>-</td>
<td>-</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>HP</td>
<td>4</td>
<td>12.77 ± 6.97</td>
<td>32.77 ± 8.94</td>
<td>14.19 ± 4.31</td>
<td>109.13 ± 55.15</td>
<td>-</td>
<td>-</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP</td>
<td>8</td>
<td>15.09 ± 8.29</td>
<td>32.77 ± 8.94</td>
<td>14.19 ± 4.31</td>
<td>109.13 ± 55.15</td>
<td>-</td>
<td>-</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HP</td>
<td>8</td>
<td>32.77 ± 8.94</td>
<td>14.19 ± 4.31</td>
<td>109.13 ± 55.15</td>
<td>-</td>
<td>-</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP</td>
<td>12</td>
<td>14.19 ± 4.31</td>
<td>109.13 ± 55.15</td>
<td>-</td>
<td>-</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HP</td>
<td>12</td>
<td>109.13 ± 55.15</td>
<td>-</td>
<td>-</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP</td>
<td>17</td>
<td>17.01 ± 5.04</td>
<td>18.24 ± 5.35</td>
<td>-</td>
<td>-</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HP</td>
<td>17</td>
<td>18.24 ± 5.35</td>
<td>-</td>
<td>-</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1NP diet contained 15% and HP diet contained 35% protein as energy
2For urinary protein and creatinine clearance(s) up to 12 months, n=8-10 per diet-time point.
3Due to metabolic cage size restrictions, 24 hour urine output was not collected for the 17 month animals and as a result, creatinine clearance could not be calculated.
**Appendix Table 8.4** Renal histological analyses of mean glomerular volume (MGV), glomerulosclerosis, and tubulointerstitial fibrosis in rats offered normal protein (NP) or high protein (HP) diets.\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>NP 4</th>
<th>HP 4</th>
<th>NP 8</th>
<th>HP 8</th>
<th>NP 12</th>
<th>HP 12</th>
<th>NP 17</th>
<th>HP 17</th>
<th>Diet P</th>
<th>Time P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGV (µm(^3) x 10(^6))</td>
<td>1.73</td>
<td>2.51</td>
<td>2.25</td>
<td>2.52</td>
<td>2.49</td>
<td>2.867</td>
<td>2.07</td>
<td>2.55</td>
<td>0.0001</td>
<td>0.0032</td>
</tr>
<tr>
<td></td>
<td>± 0.08</td>
<td>± 0.16</td>
<td>± 0.21</td>
<td>± 0.21</td>
<td>± 0.13</td>
<td>± 0.19</td>
<td>± 0.1</td>
<td>± 0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glomerulosclerosis(^2)</td>
<td>0.0127</td>
<td>0.0133</td>
<td>0.0197</td>
<td>0.0221</td>
<td>0.012</td>
<td>0.0217</td>
<td>0.0249</td>
<td>0.0322</td>
<td>0.0003</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>± 0.001</td>
<td>± 0.0016</td>
<td>± 0.0027</td>
<td>± 0.002</td>
<td>± 0.0012</td>
<td>± 0.0025</td>
<td>± 0.0014</td>
<td>± 0.0016</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubulointerstitial Fibrosis</td>
<td>0.0349</td>
<td>0.0305</td>
<td>0.0420</td>
<td>0.0344</td>
<td>0.0353</td>
<td>0.0384</td>
<td>0.0517</td>
<td>0.063</td>
<td>0.8487</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>± 0.0035</td>
<td>± 0.0027</td>
<td>± 0.0043</td>
<td>± 0.0033</td>
<td>± 0.0027</td>
<td>± 0.0045</td>
<td>± 0.0032</td>
<td>± 0.007</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)NP diet contained 15% and HP diet contained 35% protein as energy

\(^2\)There was an interaction of Diet x Time with \(P = 0.0505\).
Appendix Table 8.5 Renal disease progression and markers of renal inflammation in rats offered normal protein (NP) or high protein (HP) diets as determined by the measurement of transforming growth factor beta-1 (TGF-β₁), monocyte chemoattractant protein-1 (MCP-1), and regulated upon activation normal T-cell expressed and secreted (RANTES), respectively.¹

<table>
<thead>
<tr>
<th></th>
<th>NP 4</th>
<th>HP 4</th>
<th>NP 8</th>
<th>HP 8</th>
<th>NP 12</th>
<th>HP 12</th>
<th>NP 17</th>
<th>HP 17</th>
<th>Diet P</th>
<th>Time P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β₁ (pg/mg Renal Protein)</td>
<td>146.32 ± 17.11</td>
<td>142.22 ± 12.97</td>
<td>215.7 ± 6.38</td>
<td>206.31 ± 17.16</td>
<td>170.82 ± 6.11</td>
<td>199.87 ± 12.55</td>
<td>164.64 ± 14.88</td>
<td>170.08 ± 13.64</td>
<td>0.6469</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TGF-β₁ (pg/mg Dry Kidney)</td>
<td>68.01 ± 7.24</td>
<td>71.82 ± 5.11</td>
<td>116.13 ± 4.78</td>
<td>114.61 ± 5.78</td>
<td>92.78 ± 4.4</td>
<td>104.85 ± 4.31</td>
<td>78.93 ± 7.2</td>
<td>80.18 ± 6.82</td>
<td>0.2973</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TGF-β₁ (pg/mg Wet Kidney)</td>
<td>14.85 ± 1.58</td>
<td>15.43 ± 1.1</td>
<td>25.42 ± 1.26</td>
<td>24.67 ± 1.38</td>
<td>18.93 ± 0.98</td>
<td>21.17 ± 1.08</td>
<td>15.63 ± 1.49</td>
<td>15.38 ± 1.13</td>
<td>0.6132</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TGF-β₁ (ng/Kidney)</td>
<td>14.55 ± 1.85</td>
<td>17.32 ± 1.62</td>
<td>28.2 ± 1.2</td>
<td>31.34 ± 1.97</td>
<td>25.43 ± 1.11</td>
<td>31.63 ± 2.02</td>
<td>24.03 ± 2.58</td>
<td>28.89 ± 3.18</td>
<td>0.0043</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MCP-1 (pg/mg Renal Protein)</td>
<td>85.09 ± 5.17</td>
<td>65.71 ± 3.36</td>
<td>77.77 ± 2.38</td>
<td>69.54 ± 7.07</td>
<td>109.65 ± 4.78</td>
<td>87.68 ± 4.97</td>
<td>90.92 ± 3.8</td>
<td>80.01 ± 2.73</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MCP-1 (pg/mg Dry Kidney)¹</td>
<td>40.16 ± 2.46</td>
<td>33.8 ± 2.08</td>
<td>41.65 ± 0.88</td>
<td>38.26 ± 2.13</td>
<td>59.12 ± 2.47</td>
<td>46.32 ± 0.92</td>
<td>43.15 ± 1.28</td>
<td>37.19 ± 1.82</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MCP-1 (pg/mg Wet Kidney)</td>
<td>8.77 ± 0.54</td>
<td>7.26 ± 0.45</td>
<td>9.1 ± 0.28</td>
<td>8.22 ± 0.43</td>
<td>12.04 ± 0.63</td>
<td>9.37 ± 0.63</td>
<td>8.61 ± 0.58</td>
<td>7.15 ± 0.3</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MCP-1 (ng/Kidney)¹</td>
<td>8.53 ± 0.65</td>
<td>8.05 ± 0.53</td>
<td>10.12 ± 0.29</td>
<td>10.4 ± 0.5</td>
<td>16.26 ± 0.67</td>
<td>13.76 ± 0.61</td>
<td>13.04 ± 0.47</td>
<td>13.31 ± 0.71</td>
<td>0.1433</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RANTES (ng/mg Renal Protein)</td>
<td>396.27 ± 33.42</td>
<td>231.09 ± 34.79</td>
<td>345.55 ± 81.2</td>
<td>203.43 ± 37.4</td>
<td>538.46 ± 63.59</td>
<td>378.02 ± 26.3</td>
<td>685.12 ± 116.34</td>
<td>435.15 ± 101.51</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RANTES (ng/mg Dry Kidney)</td>
<td>377.15 ± 37.69</td>
<td>240.69 ± 35.5</td>
<td>368.31 ± 87.03</td>
<td>232.91 ± 44.17</td>
<td>577.84 ± 65.49</td>
<td>402.53 ± 31.02</td>
<td>639.02 ± 84.2</td>
<td>393.03 ± 82.2</td>
<td>&lt;0.0001</td>
<td>0.0002</td>
</tr>
<tr>
<td>RANTES (ng/mg Wet Kidney)</td>
<td>82.37 ± 8.23</td>
<td>51.7 ± 7.63</td>
<td>79.91 ± 18.04</td>
<td>49.52 ± 9.44</td>
<td>119.55 ± 13.89</td>
<td>80.78 ± 61.83</td>
<td>125.48 ± 16.68</td>
<td>76.71 ± 16.63</td>
<td>&lt;0.0001</td>
<td>0.0025</td>
</tr>
<tr>
<td>RANTES (µg/Kidney)</td>
<td>8.0 ± 8.84</td>
<td>57.27 ± 9.58</td>
<td>87.69 ± 19.38</td>
<td>63.65 ± 12.27</td>
<td>157.98 ± 16.96</td>
<td>119.1 ± 8.28</td>
<td>192.86 ± 25.45</td>
<td>142.03 ± 33.39</td>
<td>0.0019</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

¹NP diet contained 15% and HP diet contained 35% protein as energy
²There was an interaction of Diet* Time with P = 0.084, P = 0.0574, respectively.
8.6 Health Concerns

Originally the study started with 86 rats, that were randomized to 2 diets (HP and NP) with 4 time points (4, 8, 12, and 20 months). This gave an n=10 per diet-time point for the first 2 endpoints than n=11, and n=12 (n=86) for the 3rd and 4th endpoints, respectively. Due to the longevity of the study, these extra rats were maintained in case health problems arose and to maintain an n=8 and with it statistical power.

8.6.1 Twelve Month Tumors

Nine months into the study (November 24, 2005) four animals developed mammary tumors, 2 from each diet. These rats were almost a year old and were born between December 2 and 9, 2004. A fifth animal, a NP rat was discovered to have a tumor on December 1, 2005. The tumors were found at an early stage and did not interfere with ambulation, eating, drinking, or welfare issues.

However, the 3 rats on the NP diet, 35LN, 47L, and 53LN, all had large tumors that required surgery in order to meet the 12 month feeding endpoint without being a humane issue. The 2 HP rats, 54H and 70H did not require surgery based on veterinary services recommendations. Normal protein rats were numbered with odd numbers and the letter L. High protein rats had even numbers and the letter H. The letter N represented a notch on the rats ear.

On December 13, 2005, 35LN, 47L, and 53LN underwent surgery to remove the tumors. The animals were closed with staples (clips) with sutures in-between and collared. The animals, including those whose tumors were not removed were monitored closely by the animal handling staff. Those that had surgery returned to their prior healthy weight and made the 12 month termination point in mid-February.
Appendix Table 8.6.2 Tumor description of 12 month animals.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Animal ID</th>
<th>Tumor Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>35LN</td>
<td>2 cm detached tumor in right axilla area</td>
</tr>
<tr>
<td>NP</td>
<td>47L</td>
<td>2 cm detached tumor on lateral chest wall behind right axilla area</td>
</tr>
<tr>
<td>NP</td>
<td>53LN</td>
<td>3 cm detached tumor in right inguinal area</td>
</tr>
<tr>
<td>HP</td>
<td>54H</td>
<td>2 small detached tumors in right inguinal area</td>
</tr>
<tr>
<td>HP</td>
<td>70H</td>
<td>4 small detached tumors, BB size in a chain on lateral chest wall behind left axilla area</td>
</tr>
</tbody>
</table>

High Protein (HP) Diet, Normal Protein (NP) Diet.

8.6.3 Early 12 Month Euthanized Animal

On January 23, 2006 a NP rat 61LN was found to have a prolapsed bladder. The rat was euthanized prior to the 12 month end point. Post mortem revealed an anomaly of the reproductive tract. The left horn of the uterus appeared normal in size, but the right horn of the uterus became increasingly narrow caudally. The two horns joined together to form a single larger tube before attaching to the vagina. At the point where the two horns joined there was a polyp. The left horn of the uterus was patent, but unable to pass a probe into the right horn. The two cervices and vagina looked normal. The polyp was what was seen sticking out of the vagina. As it grew in size it caused some irritation that caused the rat to try and expel it. The polyp was inside the vagina and the two horns of the uterus had been everted into the vaginal canal as well.

8.6.4 Health Complications of Early Euthanized Animals

During May 2006, the HP rats 38H had lost 20% body weight within one month. Upon further examination, the rat appeared dusky in colour, weak, slightly dyspneic. This rat was observed falling over while grooming itself. This animal was euthanized on May 17, 2006 and the post-mortem revealed right sided heart failure.
HP rat 72HN was found to have very hard, lobular mass (approximately 3 cm in diameter) in the front of its left hind leg (May 25, 2006). The rat was operated on June 5, 2006 to remove the tumor, but had to be euthanized the next day due to tumor location on the abdominal wall and its negative effects on health.

NP rat 41L had lost 8% of its body weight from April 17 to May 17, 2006. By June 7, 2006, the rat had lost 19% body weight and a tumor was found near the right front leg. The rat was euthanized on June 9, 2006.

A 1 cm diameter tumor with an undefined mass at the cranial end in the left hind flank area was discovered in the NP rat 63L on June 8, 2006. By June 15, 2006, the tumor had grown to a length of 3 to 4 cm, approximately 1 cm in width, and was bi-lobed. The rat was operated on June 21, 2006 and the large necrotic tumor had its own blood vessels in the inguinal area. On June 23, 2006, the rat was found on its back, unable to right itself, and with an open wound. It was not possible to re-close the wound and the rat was euthanized.

Two tumors were found in the NP rat 77LN on June 8, 2006. The first was ¾ cm in diameter near the front left axilla, and the second was 1 cm in diameter in left flank. The rat was brought in for surgery on June 21, 2006 with a large amount of diarrhea in the cage. This animal had a history of recurrent diarrhea and experienced a 6% weight loss. Based on the rat's history, it was recommended for euthanasia. The animal was euthanized on June 27, 2006.

On April 20, 2006, a firm mass in the left inguinal area (approximately 1cm diameter) was found on rat 43L (NP rat). After 9% weight loss, the tumor was removed on May 8, 2006 and healed well up until June 8, 2006, when another 1cm diameter tumor
was discovered in the right front axilla area. A chain of several hard, "grainy" tumors was palpated on June 28, 2006 and surgery was not an option due to veterinary services policy not to operate twice on an animal, and as a result, the rat was euthanized on June 30, 2006.

8.6.5 Animals that Required Surgery to Remove Tumors to Make 17 Month Endpoint (July 5, 2006)

A soft mass (approximately 1 cm diameter) on the right flank was discovered on 56 HN (rat HP) on April 6, 2006. By April 28, 2006, the mass had grown to 2-2.5 cm in diameter and on May 8, 2006, the tumor was removed. The rat healed well, but on June 15, 2006, a second tumor was found in the right axilla (approximately ¾ cm in diameter). The rat was closely monitored for weight loss and other signs of ill health. This rat remained healthy and was terminated at the 17 month endpoint.

On May 11, 2006, a 1 cm diameter lump was observed on the left front axilla area in rat 36HN (HP). On May 17, 2006, the animal underwent surgery to remove the tumor. The animal healed well and was terminated at the 17 month endpoint.

A hard mass, less than 1 cm in diameter on the left flank was found on rat 74H (HP) on May 25, 2006. The tumor was removed on June 5, 2006 and the incision healed well. The animal was monitored closely and made the 17 month endpoint.

Hp rat 44 HN was found to have a ¾ cm diameter tumor on the right front axilla area close to the back of leg on June 8, 2006. It was not impeding mobility and did not change in size. On June 21, 2006, the tumor was removed successfully and this rat was monitored until the 17 month end-point when it was terminated.

A suspected tumor that was ¾ cm in diameter was discovered in the front axilla area on June 8, 2006, very close to leg in rat 68HN (HP). It did not impede mobility and
growth was monitored closely. The rat was operated on June 21, 2006 and the tumor out to be a hematoma. It was lanced, and the blood drained. There was some hypertrophy of the mammary tissue in the left axilla, as well as the inguinal areas. The animal handling staff monitored, but no further complications arose and the animal was terminated at the 17 month endpoint.

8.6.6 Animals that did not Undergo Tumor Removal at 17 Months

On June 22, 2006, tumors were discovered in HP rat 42H associated with the first inguinal mammary gland on the left side and NP rat 71L which had a hard, pea-sized lump about 1 cm lateral to the first inguinal mammary gland on the left side. These animals were monitored up until the 17 month endpoint when they were terminated.

**Appendix Table 8.6.7** Tumor description of 17 month animals.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Animal ID</th>
<th>Tumor Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP</td>
<td>72HN</td>
<td>3 cm lobular mass on front of left hind leg</td>
</tr>
<tr>
<td>NP</td>
<td>41L</td>
<td>Tumor on right front leg</td>
</tr>
<tr>
<td>NP</td>
<td>63L</td>
<td>3 to 4 cm bi-lobed tumor at cranial end in the left hind flank</td>
</tr>
<tr>
<td>NP</td>
<td>77LN</td>
<td>2 small tumors, ¾ cm on front left axilla area, and 1 cm on left flank</td>
</tr>
<tr>
<td>NP</td>
<td>43L</td>
<td>1 cm firm mass in left inguinal area, a 2nd 1cm tumor on right front axilla area, and a chain of several hard, &quot;grainy&quot; tumors</td>
</tr>
<tr>
<td>HP</td>
<td>56HN</td>
<td>2-2.5 cm soft mass on right flank, a 2nd ¾ cm tumor on right axilla area</td>
</tr>
<tr>
<td>HP</td>
<td>36HN</td>
<td>1 cm lump left front axilla area</td>
</tr>
<tr>
<td>HP</td>
<td>74H</td>
<td>1 cm hard mass on left flank</td>
</tr>
<tr>
<td>HP</td>
<td>44HN</td>
<td>¾ cm tumor on right front axilla on back of leg</td>
</tr>
<tr>
<td>HP</td>
<td>68HN</td>
<td>¾ cm on front axilla area close to leg, was actually hematoma</td>
</tr>
<tr>
<td>NP</td>
<td>42H</td>
<td>Tumor associated with 1st inguinal mammary gland on left side</td>
</tr>
<tr>
<td>NP</td>
<td>71L</td>
<td>1 cm hard pea sized lump also on 1st inguinal mammary gland on left side</td>
</tr>
</tbody>
</table>

High Protein (HP) Diet, Normal Protein (NP) Diet.

8.6.8 Animals that were Excluded for Statistical Reanalysis

The rats excluded from these analyses at 12 months were: 35LN, 47L, 53LN, 54H, and 70H. The following animals were excluded for the 17 month endpoint analyses:
36HN, 44HN, 42H, 56HN, 68HN, 74H, 23LN, 39LN, and 71L. Although NP rats 23LN and 39LN had not developed tumors, they were excluded from the analyses due to 4% and 8% weight loss prior to the 17 month endpoint.

### 8.7 Body Composition Excluding Animals with Health Concerns Results

Firstly, rats that consumed HP had body weights that were ~8% lower than NP (399.32 ± 14.88 g versus 434.96 ± 18.19 g, respectively, P = 0.0104, Appendix Figure 8.7.1). Similar to body weight, percent body fat was ~37% lower in animals on HP compared to the NP diet (24.09 ± 1.82 versus 33.02 ± 1.94, respectively, P < 0.0001, Appendix Figure 8.7.2). Following suit with a reduction in body fat was a significant increase in lean body mass by 13% in the HP rats (73.55 ± 1.71 % compared to 64.28 ± 1.77 % in NP, P < 0.0001, Appendix Figure 8.7.3).

A comparison table of the diet-time point means and SEMs for body composition before and after removal of animals with health concerns is located in the appendix (Appendix Table 8.12). Statistically, these parameters did not differ significantly upon removal of the animals with health concerns.
Appendix Figure 8.7.2 Body weights. Data is presented as mean ± SEM (n = 5-10). 2X4 ANOVA with P <0.05 considered significantly different. Diet, P = 0.0104 and Time, P <0.0001.

Appendix Figure 8.7.3 Percent body fat. Diet, P <0.0001 and Time, P <0.0001.
Appendix Figure 8.7.4 Percent lean weight. Diet, $P < 0.0001$ and Time, $P < 0.0001$. 
8.8 Kidney Weights Excluding Animals with Health Concerns

Kidney weights were ~13% higher in rats that were offered the HP diet compared to NP (1.37 ± 0.04 g versus 1.22 ± 0.05 g, respectively, P = 0.0004, Appendix Figure 8.8.1). Since the HP animals weighed less and had increased lean muscle mass, kidney weights were also expressed based on body weight (BW) and lean body weight (LBW). Renal hypertrophy remained with HP feeding with kidney weights that were ~23% higher when expressed per body weight (0.70 ± 0.017 g kidney weight/100g BW versus 0.57 ± 0.011 kidney weight/100g BW, P < 0.0001, Appendix Figure 8.8.2). There was a trend towards higher kidney weights per gram lean body weight in HP compared to the NP diet, although it was not significant (0.96 ± 0.026 g kidney weight/100g LBW versus 0.90 ± 0.025 g kidney weight/100g LBW, P = 0.0593, respectively, Appendix Figure 8.8.3).

The kidneys of the HP had ~16% higher levels of protein content compared to the NP group (147.21 ± 5.95 mg/kidney versus 127.13 ± 4.34 mg/kidney, respectively, P = 0.0023, Appendix Figure 8.8.4). When expressed per gram of kidney, renal protein content was ~8% lower in the HP rats compared to the NP rats (4.12 ± 0.22 mg/g kidney versus 4.47 ± 0.18 mg/g kidney, respectively, P = 0.0488, Appendix Figure 8.8.5).

Besides the lack of significance in kidney weights when expressed based on lean mass (P = 0.0593 from P = 0.0023), the exclusion of animals with health concerns did not change the effect of HP on renal hypertrophy. For a detailed comparison between means, SEMs, and P values see Appendix Table 8.12.
Appendix Figure 8.8.1 Kidney weights. Diet, P = 0.0004 and Time, P < 0.0001.

Appendix Figure 8.8.2 Kidney weights per body weight. Diet, P < 0.0001 and Time, P = 0.9245.
Appendix Figure 8.8.3 Kidney weights per lean body weight. Diet, $P = 0.0593$ and Time, $P < 0.0001$.

Appendix Figure 8.8.4 Renal protein content per kidney. Diet, $P = 0.0023$ and Time, $P < 0.0001$. 
Appendix Figure 8.8.5 Renal protein content per gram of kidney. Diet, $P = 0.0488$ and Time, $P < 0.0001$. 
8.9 Renal Function Excluding Animals with Health Concerns

Urinary protein excretion per 24 hours was ~4.8 times higher in HP compared to NP rats (51.55 ± 19.68 mg/24 hr versus 10.68 ± 17.42 mg/24, respectively, P <0.0001, Appendix Figure 8.9.1).

Urinary protein per urinary creatinine was used up to the 17 month time point since bladder urine was collected at termination. Once again those on the HP diet had ~3.5 times higher urinary protein/urinary creatinine than NP (3.84 ± 1.36 versus 1.09 ± 0.33, respectively, P = 0.0002, Appendix Figure 8.9.2).

There was a significant effect of the HP diet on creatinine clearance compared to NP. The clearance values for the HP animals was ~20% higher (1.33 ± 0.088 ml/min versus 1.11 ± 0.099 ml/min, P = 0.0453, Appendix 8.9.3). When creatinine clearance was corrected for body weight, hyperfiltration was also ~29% higher in HP rats compared to NP (0.36 ± 0.025 ml/min, versus 0.28 ± 0.025 ml/min, P = 0.0005, Appendix Figure 8.9.4). However, when creatinine clearance was corrected for lean body weight, no effect of diet was seen (HP 0.48 ± 0.033 ml/min, NP 0.41 ± 0.035 ml/min, P = 0.0774, Appendix Figure 8.9.5).

Removal of animals with health concerns increased the effect of the HP diet on creatinine clearance to significance (P = 0.0453 from P = 0.055). Otherwise the effect of HP diet on renal function remained similar (Appendix Table 8.12).
Appendix Figure 8.9.1 Proteinuria excluding animals. Data is presented as mean ± SEM (n = 7-10). 2X3 ANOVA with P <0.05 considered significantly different. Diet, P <0.0001 and Time, P <0.0001.

Appendix Figure 8.9.2 Urinary protein per urinary creatinine (n = 4-10). Diet, P = 0.0002 and Time, P <0.0001.
Appendix Figure 8.9.3 Creatinine clearance (n = 5-10). Diet, P = 0.0453 and Time, P = 0.3882.

Appendix Figure 8.9.4 Creatinine clearance per 100 g BW. Diet, P = 0.0005 and Time, P = 0.3451.
Appendix Figure 8.9.5 Creatinine clearance per 100 g LBW. Diet, \( P = 0.0773 \) and Time, \( P = 0.6912 \).
8.10 Renal Histology Excluding Animals with Health Concerns

MGV was 21% higher with HP feeding than animals on NP (2.57 ± 0.1 μm$^3$ x 10$^6$ versus 2.12 ± 0.09 μm$^3$ x 10$^6$, respectively, P = 0.0012, Appendix Figure 8.10.1).

Animals on the HP diet had 29% more glomerulosclerosis when compared to NP (0.0211 ± 0.0015 versus 0.0164 ± 0.0013, respectively, P = 0.0006, Appendix Figure 8.10.2). There was however, an interaction between diet and time at P = 0.0431 for glomerulosclerosis. Through contrasts it was found that glomerulosclerosis occurred by 12 months of age in the HP rats.

When sclerosis of the renal cortex including glomeruli, tubules, and the interstitial area was measured, no effect of diet was seen between groups (HP 0.038 ± 0.003 versus NP 0.03945 ± 0.002, P = 0.9268, Appendix Figure 8.10.3).

Statistically, renal histology did not differ significantly upon removal of the animals with health concerns. A comprehensive comparison between results with and without these animals can be found in Appendix Figure 8.12.
Appendix Figure 8.10.1 Mean glomerular volume (n = 5-10). Diet, P = 0.0012 and Time, P = 0.0045.

Appendix Figure 8.10.2 Glomerulosclerosis. Diet, P = 0.0006, Time, P < 0.001, and Diet x Time P = 0.0431. *Contrasts demonstrated that glomerulosclerosis occurred by 12 months.
Appendix Figure 8.10.3 Tubulointerstitial fibrosis. Diet, P = 0.9268 and Time, P = 0.0002.
8.11 Inflammatory Proteins Excluding Animals with Health Concerns

There was no significant statistical difference in TGF-β₁ between diets when expressed based on renal protein content (HP 177.84 ± 8.76 pg/mg renal protein versus NP 171.9 ± 8.00 pg/renal protein, P = 0.4367, Appendix Figure 8.11.1). When expressed as total TGF-β₁ per kidney, HP animals had levels of TGF-β₁ that were ~18 % higher than NP (25971.01 ± 1456.31 pg/kidney versus 22051.68 ± 1281.32 pg/kidney, respectively, P = 0.0056, Appendix Figure 8.11.2). There was no significant difference between dietary treatments when the amount of TGF-β₁ was expressed per mg dry kidney (HP 92.51 ± 4.25 pg/mg dry kidney versus NP 88.27 ± 4.78 pg/mg dry kidney, P = 0.3195, Appendix Figure 8.11.3). The same could be said when TGF-β₁ was expressed per mg wet kidney (HP 19.34 ± 0.96 pg/mg wet kidney versus NP 18.74 ± 1.11 pg/mg wet kidney, P = 0.483, Appendix Figure 8.11.4)

Surprisingly, animals offered HP had ~17% and ~43% lower MCP-1 and RANTES levels than NP when expressed based on renal protein (74.27 ± 3.17 pg MCP-1/mg renal protein versus 89.56 ± 3.13 pg MCP-1/mg renal protein, respectively, P = 0.0001, Appendix Figure 8.11.5; 268.66 ± 21.86 ng RANTES/mg renal protein versus 470.67 ± 48.9 ng RANTES/mg renal protein, respectively, P <0.0001, Appendix Figure 8.11.9).

When the data was expressed per whole kidney, those offered the HP diet had 8% lower levels of MCP-1 (10.66 ± 4.92 pg/kidney versus 11.51 ± 6.41 ng/kidney, respectively, P = 0.0258, Appendix Figure 8.11.6). There was an interaction of Diet x Time P = 0.0162 and contrasts showed that HP animals had significantly lower levels of MCP-1 per kidney at 12 months.
As for RANTES, the kidneys of HP rats had ~35% lower levels than NP (77.61 ± 6.56 µg/kidney versus 118.92 ± 12.9 µg/kidney, respectively, \( P = 0.0002 \), Appendix Figure 8.11.10).

Animals that consumed HP had ~16% lower levels of MCP-1 than NP per mg dry kidney (38.52 ± 1.41 pg/mg dry kidney versus 45.63 ± 1.76 pg/mg dry kidney, respectively, \( P < 0.0001 \), Appendix Figure 8.11.7) and ~17% lower levels of MCP-1 per mg wet kidney (8.03 ± 0.31 pg/mg wet kidney versus 9.60 ± 0.35 pg/mg wet kidney, respectively, \( P < 0.0001 \), Appendix Figure 8.11.8). There was an interaction of Diet x Time in renal levels of MCP-1 per mg dry tissue (\( P = 0.0486 \)). Contrasts showed that at 12 months HP had significantly lower levels of this inflammatory protein.

RANTES in the kidneys of HP animals were ~40% lower than NP per mg dry kidney (279.82 ± 22.41 ng/mg dry kidney versus 465.98 ± 42.52 ng/mg dry kidney, respectively, \( P < 0.0001 \), Appendix Figure 8.11.11) and ~35% lower levels of RANTES per mg wet kidney (57.68 ± 4.53 ng/mg wet kidney versus 97.61 ± 8.53 ng/mg wet kidney, respectively, \( P < 0.0001 \), Appendix Figure 8.11.12).

Removal of animals with health concerns decreased the levels of MCP-1 per kidney in HP rats to statistical significance (\( P = 0.0258 \) from \( P = 0.1433 \)). Otherwise the effect of HP on renal progression measured by TGF-\( \beta_1 \) and renal inflammation through MCP-1 and RANTES remained statistically similar. A more detailed comparison can be found in Appendix Table 8.12.
**Appendix Figure 8.11.1** Levels of renal TGF-β₁ per mg protein. *(n = 5-10).* Diet, *P* = 0.4367 and Time, *P* < 0.0001.

**Appendix Figure 8.11.2** Levels of TGF-β₁ per kidney. Diet, *P* = 0.0056 and Time, *P* < 0.0001.
Appendix Figure 8.11.3 Levels of TGF-β1 per mg dry kidney. Diet, $P = 0.3195$ and Time, $P < 0.0001$.

Appendix Figure 8.11.4 Levels of TGF-β1 per mg wet kidney. Diet, $P = 0.483$ and Time, $P < 0.0001$.  

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Appendix Figure 8.11.5 Levels of renal MCP-1 per mg protein (n = 4-10). Diet, P = 0.0001 and Time, P < 0.0001.

Appendix Figure 8.11.6 Levels of MCP-1 per kidney. Diet, P = 0.0258, Time, P < 0.0001, and Diet x Time, 0.0162. *Contrasts showed that MCP-1 levels were significantly lower in HP kidneys at 12 months.
Appendix Figure 8.11.7 Levels of MCP-1 per dry kidney. Diet, P < 0.0001, Time, P < 0.0001, and Diet x Time, P = 0.0486. *Contrasts showed that MCP-1 levels were significantly lower in HP kidneys at 12 months.

Appendix Figure 8.11.8 Levels of MCP-1 per wet kidney. Diet, P < 0.0001 and Time, P < 0.0001.
Appendix Figure 8.11.9 Levels of renal RANTES per mg protein. Diet, P <0.0001 and Time, P = 0.0008.

Appendix Figure 8.11.10 Levels of RANTES per kidney. Diet, P = 0.0002 and Time, P <0.0001.
**Appendix Figure 8.11.11** Levels of RANTES per dry kidney. Diet, $P < 0.0001$ and Time, $P = 0.0033$.

**Appendix Figure 8.11.12** Levels of RANTES per wet kidney. Diet, $P < 0.0001$ and Time, $P = 0.026$. 
Appendix Table 8.12 Comparison between statistical analyses of all parameters at 12 and 17 months after animals with health concerns were omitted from the results.\(^1,2\)

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<tr>
<th></th>
<th>NP 12</th>
<th>NP 12</th>
<th>HP 12</th>
<th>HP 12</th>
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<th>HP 17</th>
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<th>Diet P</th>
<th>Time P</th>
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<td>MGV (µm³ x 10⁶)</td>
<td>Glomerulosclerosis¹</td>
<td>Tubulointerstitial Fibrosis</td>
<td>TGF-β1 (pg/mg Renal Protein)</td>
<td>TGF-β1 (pg/mg Dry Kidney)</td>
<td>TGF-β1 (pg/mg Wet Kidney)</td>
<td>MCP-1 (pg/mg Renal Protein)</td>
<td>MCP-1 (pg/mg Dry Kidney)²</td>
<td>MCP-1 (pg/mg Wet Kidney)</td>
<td>RANTES (ng/mg Renal Protein)</td>
<td>RANTES (ng/mg Dry Kidney)</td>
<td>RANTES (ng/mg Wet Kidney)</td>
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<td>2.49</td>
<td>± 0.13</td>
<td>0.012 ± 0.0001</td>
<td>0.035 ± 0.0027</td>
<td>170.82 ± 6.11</td>
<td>18.93 ± 0.98</td>
<td>109.65 ± 5.06</td>
<td>59.12 ± 2.13</td>
<td>12.04 ± 0.41</td>
<td>538.46 ± 63.59</td>
<td>577.83 ± 65.49</td>
<td>119.55 ± 13.89</td>
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<td>± 0.16</td>
<td>0.0113 ± 0.0005</td>
<td>0.0329 ± 0.0034</td>
<td>163.15 ± 6.57</td>
<td>18.55 ± 1.3</td>
<td>112.1 ± 5.25</td>
<td>61.49 ± 2.12</td>
<td>12.56 ± 0.27</td>
<td>462.78 ± 78.85</td>
<td>503.06 ± 73.8</td>
<td>106.26 ± 17.36</td>
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<td>2.87</td>
<td>± 0.19</td>
<td>0.0217 ± 0.0025</td>
<td>0.0384 ± 0.0045</td>
<td>199.87 ± 10.45</td>
<td>21.17 ± 1.08</td>
<td>87.68 ± 4.97</td>
<td>46.32 ± 2.47</td>
<td>9.37 ± 0.83</td>
<td>378.02 ± 26.3</td>
<td>402.53 ± 31.11</td>
<td>80.78 ± 3.11</td>
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<td>± 0.23</td>
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<td>103.47 ± 5.01</td>
<td>20.85 ± 1.27</td>
<td>89.32 ± 4.97</td>
<td>46.49 ± 2.84</td>
<td>9.39 ± 0.74</td>
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<td>383.99 ± 35.46</td>
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<td>78.93 ± 7.2</td>
<td>15.63 ± 1.49</td>
<td>90.92 ± 4.12</td>
<td>43.15 ± 0.92</td>
<td>8.61 ± 0.58</td>
<td>685.12 ± 116.34</td>
<td>639.02 ± 84.2</td>
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<td>0.0322 ± 0.0043</td>
<td>0.0572 ± 0.0063</td>
<td>70.27 ± 7.57</td>
<td>13.4 ± 1.35</td>
<td>90.53 ± 4.12</td>
<td>42.29 ± 0.4</td>
<td>8.1 ± 0.41</td>
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<td>15.38 ± 1.13</td>
<td>80.01 ± 5.28</td>
<td>37.19 ± 1.28</td>
<td>7.15 ± 0.61</td>
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<td>147.67 ± 14.88</td>
<td>76.71 ± 20.93</td>
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<td>72.18 ± 2.79</td>
<td>14.11 ± 0.85</td>
<td>77.4 ± 5.01</td>
<td>35 ± 2.11</td>
<td>6.76 ± 0.61</td>
<td>170.08 ± 13.64</td>
<td>170.08 ± 13.64</td>
<td>63 ± 4.92</td>
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<td>155 ± 3.39</td>
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<td>101.5 ± 5.01</td>
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<td>0.6469 ± 0.4367</td>
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<td>0.61 ± 0.31</td>
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<td>0.8487 ± 0.9268</td>
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<td>0.84 ± 0.27</td>
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</table>
1 n=4-11 per diet-time point
2 Since no animals were excluded at 4 and 8 months due to health complications, the P values listed include these 2 time points.
3 Due to metabolic cage size restrictions, 24 hour urine output was not calculated for the 17 month animals and as a result creatinine clearance could not be calculated.
4 There was an interaction of Diet x Time with P = 0.0505 and 0.0431 when animals with health concerns were removed.
5 There was an interaction of Diet x Time with P = 0.0840 and 0.0486 when animals with health concerns were removed.
6 There was an interaction of Diet x Time with P = 0.0574 and 0.0162 when animals with health concerns were removed.